Factors Affecting Plasma Viremia and Host Immune Responses in Dengue Infection

Thesis

FACTORS AFFECTING PLASMA VIREMIA AND HOST IMMUNE RESPONSES IN DENGUE INFECTION

by

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Abstract

Dengue is the most important arboviral disease affecting humans. Although mortality is generally low, dengue generates a significant burden for health services in endemic countries. Pathogenesis is poorly understood. Secondary dengue is one important risk factor for severe disease, but current algorithms to differentiate primary from secondary infections are of limited utility. Viral burden is also thought to be a major factor influencing disease severity, but existing knowledge of the magnitude and kinetics of plasma viremia in association with different dengue serotypes, primary versus secondary immune status, and/or clinical markers of disease severity, is limited and inconsistent.

I first set out to establish novel algorithms that permit differentiation of primary from secondary dengue at any day of illness with high accuracy. Using serial samples from 249 well-characterized patients, categorized as having primary or secondary infections on the basis of PRNTs after six months, I developed a number of models; the algorithm based on the Panbio Indirect IgG performed best (accuracy, 85%), although the models based on the in-house IgG and in-house IgM/IgG ratio were also good (accuracy, 84%). I subsequently applied the IgM/IgG ratio algorithm to define the immune status of 884 patients previously enrolled into early dengue studies. Using serial daily plasma samples I describe detailed plasma viremia responses for 581 DENV1, 152 DENV2 and 151 DENV3 infections. In general DENV1 infections resulted in higher plasma viremia than did DENV2 or DENV3 infections. Overall viremia was higher in primary than secondary DENV1 and DENV3 infections, although similar in DENV2 primary and secondary infections. In addition, higher viremia on Day3 of illness was significantly associated with several disease severity markers: lower platelet nadir, higher haemoconcentration and an increased risk of developing shock and bleeding.

Finally I evaluated the effect of early prednisolone therapy on plasma viremia and immune responses, using serial samples from a clinical trial assessing the safety of early oral corticosteroid therapy. Prednisolone did not augment plasma viremia or NS1 antigenaemia, confirming the safety of using the drug during active viral replication. However, the effects of the drug on a range of immune correlates was also limited, consistent with prednisolone having negligible measurable benefits in the clinical trial on which this work was based.

Authorship

The work presented here was done primarily by me, but with contributions from some other people as described below. My supervisors provided extensive training and supervision throughout the time I did my PhD program.

For most of my laboratory studies I used plasma samples and clinical information collected as part of previous and/or ongoing studies supervised by members of the Dengue Research Group at OUCRU. Although I coordinated the patient enrolment and collection of the follow-up samples (6 months after fever onset) for the study to develop the algorithms to differentiate primary from secondary dengue (Chapter 3), other aspects of the clinical studies were coordinated by other members of the research group over a number of years.

I carried out all the laboratory work presented in this thesis with the following exceptions: 1) The PRNT assays (Chapter 3) were done at the laboratory of Dr Steve Whitehead, at the National Institute of Allergy and Infectious Diseases, NIH, USA. 2) Approximately 20% of the RT-PCR reactions on the daily specimens for the work described in Chapter 4 were done by my colleague, Huynh Thi Le Duyen. The serodiagnostics (in-house IgM/IgG capture ELISAs) for the patients involved in the various studies were done by a number of different colleagues at intervals over the two years that these studies were running. 3) For the work described in Chapter 5, I carried out all the laboratory work performed in Singapore, under the supervision of Professor Martin Hibberd, but my colleagues in the Dengue Group at OUCRU helped me with the serology assays, T cell phenotyping, and cytokine measurements.

For the statistical analysis and model development I worked under close supervision by Dr Marcel Wolbers (Head of Biostatistics) and with considerable support from Dr Phung Khanh Lam (PhD student in the Biostatistics group) - I carried out the main bulk of the analyses myself but with their help. However the statistical analysis on virological parameters (viremia and NS1) described in Chapter 5 was done specifically by Dr Marcel Wolbers, not by me.

Publications

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4. **Hanh Tien NT**, Lam PK, Duyen HT, Ngoc TV, Ha PT, Kieu NT, Simmons C, Wolbers M, Wills B. *Assessment of microalbuminuria for early diagnosis and risk prediction in dengue infections*. PLoS One. 2013. 8(1):e54538.
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I am greatly indebted to the doctors and nurses at the Hospital for Tropical Diseases and Dr Ngoc's clinic, who were involved in dengue studies. Without their hard work, I could not have such a large number of samples for my thesis. Special thanks also go to the patients for their consent to take part in the studies.

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Finally, I would like to say thank to my family for being by my side all the time.

Abbreviations

95% CI	95% Confident Interval
ADE	Antibody Dependent Enhancement
AUC	Area Under Curve
cDNA	Complement Deoxyribonucleic Acid
cRNA	Complement Ribonucleic Acid
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-Specific Intercellular Adhesion Molecule 3-Grabbing Nonintegrin
DENV	Dengue Virus
DF	Dengue Fever
DHF	Dengue Haemorrhagic Fever
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide Triphosphate
DOI	Day of Illness
DSS	Dengue Shock Syndrome
EAV	Equine Arteritis Virus
EDTA	Ethylene Diamine Triacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
Fw - Rev primers	Forward – Reverse primers
GO	Gene Ontology
HCT	Hematocrit
HI	Heamaglutination Inhibition
HLA	Human Leukocyte Antigen
HTD	Hospital for Tropical Diseases
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin

IPA	Ingenuity Pathway Analysis
IQR	Inter-quartile Range
JEV	Japanese Encephalitis Virus
Kb	Kilobase-pair
NK cell	Natural Killer Cell
NPV	Negative Predictive Value
NS1	Non-structural Protein 1
OD	Optical Density
OR	Odds Ratio
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PLT	Platelet count
PPV	Positive Predictive Value
ROC	Receive Operating Characteristics
PRNT	Plaque Reduction Neutralization Test
RNA	Ribonucleic Acid
RNase	Ribonuclease
Rpm	Round per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TNF	Tumor Necrosis Factor
WHO	World Health Organization

Table of Contents

List of Figures

List of Tables

Chapter 1

INTRODUCTION

1.1 Dengue

Dengue is the most important arboviral disease affecting humans, and represents a major burden for healthcare services in tropical regions of the world. It can be caused by any one of four serotypes of dengue virus (DENV) and is transmitted by mosquito vectors, primarily *Aedes aegypti.* Dengue is also known as breakbone fever and is usually characterized by symptoms such as fever, headache, muscle and joint pains and skin rash. However, the clinical manifestations vary from a mild self-limited febrile illness, through to severe and occasionally life threatening disease. Complications include an unusual plasma leakage syndrome that may result in hypovolaemic shock, coagulation disturbances sometimes accompanied by bleeding, and organ impairment.

The first record of dengue can be found in a Chinese encyclopedia of disease symptoms and remedies that was published during the Chin Dynasty (265 to 420 AD) (Gubler 1998). Several epidemics of possible dengue were reported in French West Indies (1635), Panama (1699), Indonesia (1779), Egypt (1780) and Philadelphia (1780). During 1779-1780, major epidemics of a disease consistent with dengue occurred across Asia, Africa and North America. Following this, large epidemics were occasionally recorded until World War 2. However, the ecological disruption that occurred during the Second World War allowed dengue to spread and become endemic in many regions. The world's first recorded epidemic of severe dengue (dengue hemorrhagic fever – DHF) occurred in the Philippines from 1953 to 1954. Since then, epidemics of DHF have been reported from many regions of Asia, America and Africa [2, 3].

Dengue has now become a major global public health concern. Figure 1-1 shows annual dengue incidence and the number of countries where dengue was recorded from 1955 to 2007 as reported by the World Health Organization (WHO) [1]. The

spread of dengue is also been in Figure 1-2, which shows a map of dengue serotypes notified from 1943 – 2013 [4]. In 2008, more than 1.2 million cases were officially reported across the Americas, South East Asia and the Western Pacific. This increased to over 2.3 million in 2010. It has been estimated that there are about 50-100 million cases of dengue infection annually, with 2.5 billion people (over 40% of the world's population) at risk [http://www.who.int/mediacentre/factsheets/fs117/en/]. However, these figures might substantially underestimate the disease burden. Using modeling, Bhatt et al. estimate there to be 390 million dengue infections per year, of which 96 million are symptomatic [5]. Very little data on case fatality rates (CFR) is available but, when available, CFR varies by geographical location, depending mainly on the level of monitoring available, as well as on the training and experience of the clinicians responsible for management. In Indonesia, India and Myanmar rates of 3-5% are reported, while very low mortality (0.2%) has been reported from Thailand [1].

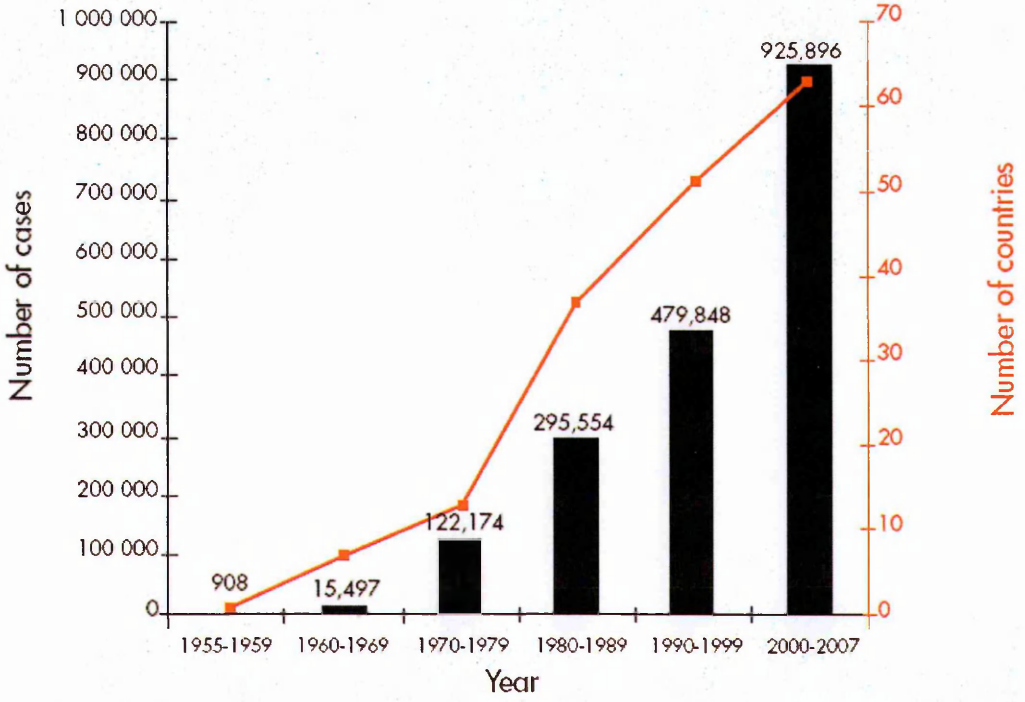


Figure 1-1: Global burden of dengue. The bars show the annual numbers of dengue cases and the line shows the number of countries reporting dengue [1]

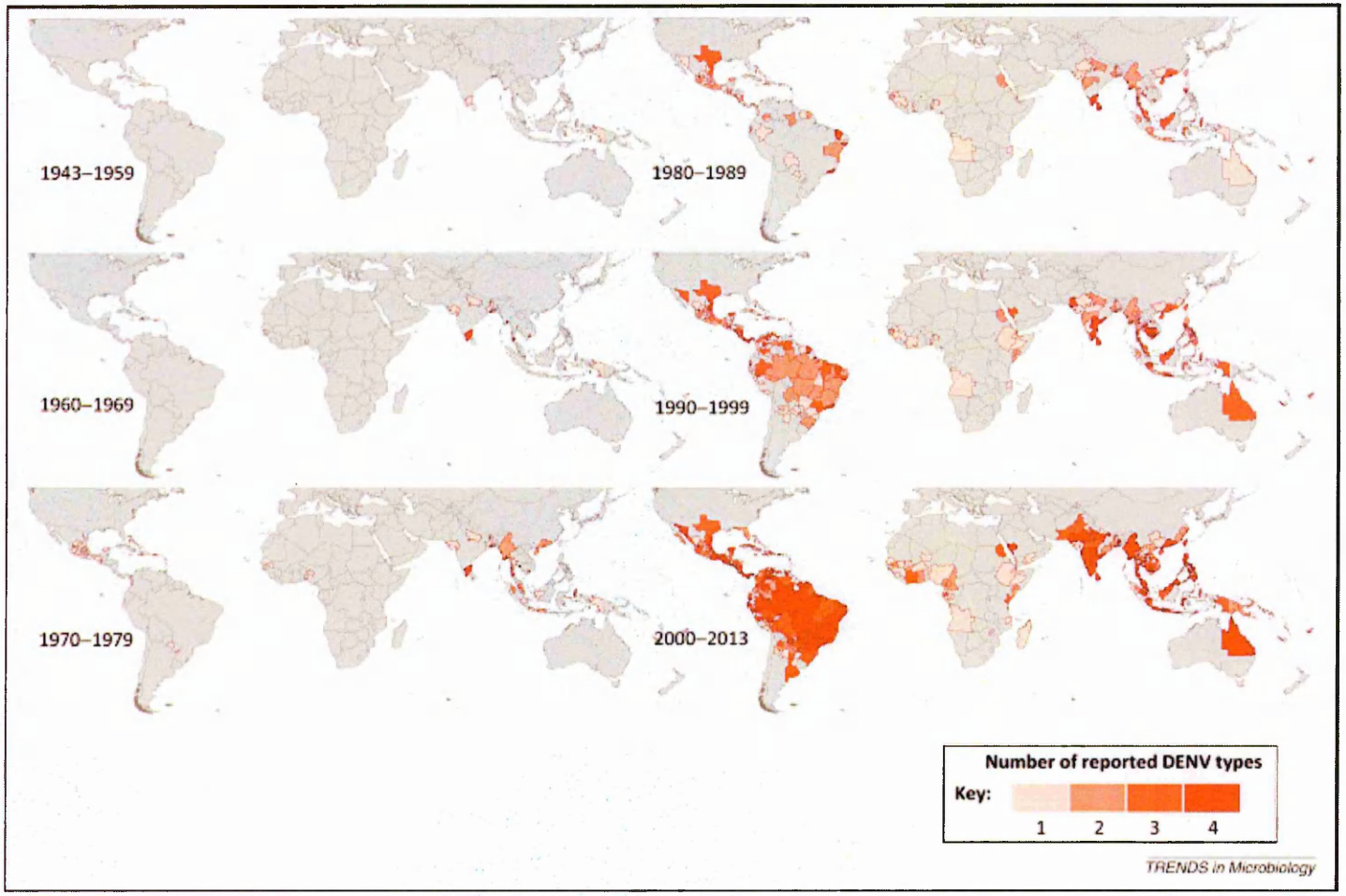


Figure 1-2: Spread of dengue serotypes from 1943-2013 [4]

1.2 Dengue epidemiology in Vietnam

Dengue was first notified in Vietnam in 1959. Because of the difference in climate patterns between the north and the south of Vietnam, dengue status is different in the two regions. With cooler weather, dengue ha s not been a major concern in the north until recently when dengue case numbers began to increase and large outbreaks were recorded, notably a very large outbreak in 2009 [6]. The status is more serious in the south. Between 2001 and 2010, 592,938 dengue cases were notified from 19 provinces of the south of Vietnam [7], and on average more than 59,000 cases were reported annually. Between 1996 and 2009, 132,480 patients were hospitalized to one of three main hospitals (Hospital for Tropical Diseases, Children's Hospital No1 and Children's Hospital No2) in Ho Chi Minh City and diagnosed with dengue. Among them, there were a total of 14,079 dengue shock syndrome (DSS) cases, most of which were children (96.6%). The case fatality rate (CFR) was higher in DSS patients compared to non-DSS patients. Fortunately however, there has been a clearly decreasing trend in CFR in the last decade from 0.64% in 1996 to 0.10% in 2004 [8]. Though the number of deaths caused by dengue is relatively small, the high number of patients requiring assessment, and potentially hospitalization for supportive management, not only results in a major burden for health service systems in Vietnam and other countries endemic for dengue, but also represents a significant drain on the national economies of these countries.

Figure 1-3 shows the upward trend of notified dengue cases in the last few decades for both the north and the south of Vietnam. However it is also important to note that dengue has been recorded throughout the country, including from the central highlands and along the coastal provinces [9-11]. Although cases are recorded dispersed through the year, many cases occur during the rainy season when the transmission season varies somewhat among the different regions and provinces, in line with different weather patterns [7].

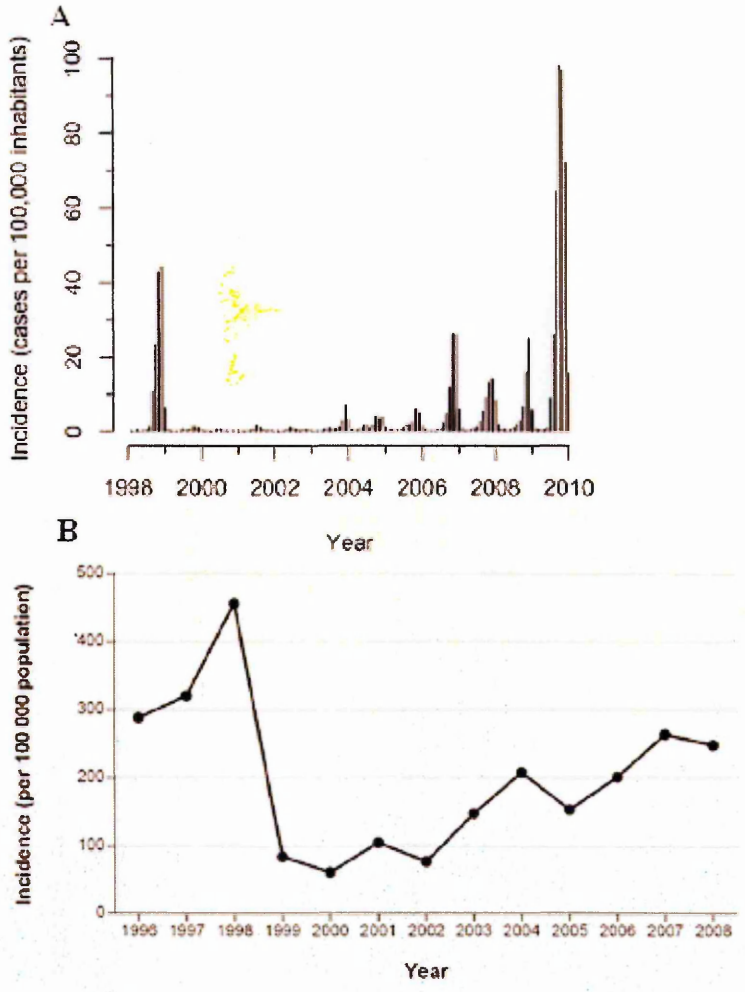


Figure 1-3: Incidence of dengue in Vietnam [6, 8]. Graph A shows the incidence of notified dengue cases in Hanoi from 1998 to 2009. Graph B shows the annual incidence of hospitalized dengue in 20 provinces of southern Vietnam from 1996 to 2008.

1.3 Dengue virus

Dengue virus is a positive sense, single stranded RNA virus, belonging to the genus *Flavivirus* of the *Flaviviridae* family. The dengue genome is approximately 11kb. It encodes three structural proteins: capside (C), membrane (M) and envelop (E); and seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5. The structural proteins are necessary components of dengue virions and the non-structural proteins have functions related to virus replication.

Although dengue antigens have been identified in a variety of human tissues such as liver, kidney, brain, skin, lung, spleen, heart and kidney, virus infection has only been confirmed in peripheral lymphocytes and macrophage and reactive splenic lymphoid cells [12-18].

Virus entry begins with the attachment of a virion to surface receptors of host cells, and then the virus penetrates the host cell mainly via clathrin mediated endocytosis [19-22]. Several molecules have been reported as receptors of DENV so far, for example C-type lectins such as dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) and C-type lectin domain family 5 member A (CLEC5A), mannose receptor, glucose-regulating protein 78 (GRP78/Bip), CD14, heparan sulfate (HS), and glycosphingolipids such as neolactotetraosylceramide (nLc_4CER) [22-29]. Briefly, receptor-bound virus is taken up by clathrin coated vesicles, which then deliver to early endosomes which take the virus to late endosomes. Due to the low pH conditions of the endosomes, the viral E glycoprotein changes its conformation and the E-homodimer dissociates and generates a fusion loop which then inserts into the target cell membrane and forms a bridge between the virus and the host cell. E protein trimers then fold into a hairpin-like structure, thus bending the target membrane towards the viral membrane and leading to fusion. Following fusion, viral RNA is released into the host cell cytoplasm and begins the process of replication and translation [22]. In vitro, dengue was found to form plaques on mammalian cells. However, in vivo, the virus does not lyse target cells to release virions; instead, after assembly, dengue virions are secreted via a process of exocytosis [30].

1.4 Mosquito vectors and transmission

Aedes aegypti is the main vector for the dengue virus, although *Aedes albopictus* has been reported to transmit dengue in some regions. These mosquitoes have adapted to live in peri-urban and urban environments where humans are their main feeding target. They lay eggs in man-made containers which may fill with rainwater. These eggs then take about 7-14 days to progress through the larval and pupal stages to become adult mosquitoes. *Aedes aegypti* usually feed in the mid-morning and late

afternoon. They become infectious when they bite infected patients with a significant viremia in their blood [31]. The virus replicates in the mosquito's mid-gut before spreading throughout the mosquito's body including to the saliva after 7-12 days [32]. After this time, it can be transmitted to healthy humans if an infectious mosquito bites them. Although vertical transmission has been demonstrated in a laboratory setting, this is rarely seen in the field. The potential for mosquitoes to transmit the virus can be influenced by changes in climate or environmental conditions, the interaction between populations of humans and mosquitoes, and the immunological status of the human population [11, 33-35].

1.5 Dengue pathogenesis

Although a number of animal models for severe dengue exist, the relationships to human clinical disease are uncertain, and thus the findings from studies using these models must be interpreted with caution. As a result our understanding of dengue pathogenesis remains limited. However, similar to other infectious diseases, it is thought that the interaction of pathogen related factors and host factors, including the host immune response and genetic background, are all important. Since the severe manifestations of dengue occur relatively late in the disease course, often when the infecting virus is no longer detectable in plasma, immune mediated mechanisms are considered to play a significant role in pathogenesis. Factors that are thought to be relevant in dengue pathogenesis are summarized in this section and in Table 1.1.

1.5.1 Viral factors

1.5.1.1 Viremia

Currently, the viremia kinetics of dengue virus is still not clear, especially in the very early phase of illness around the time of fever onset. Plasma viremia is thought to peak early in the illness course, subsequently decreasing to undetectable levels by about day 5-6, sometimes up to day 8, of illness [36]. Conventionally, primary dengue infections (the first time that an individual is infected with one of the four serotypes)

are said to be associated with lower plasma viremia than secondary infections (the second or subsequent time that an individual is infected, with a different serotype from the one that caused their primary infection). Higher plasma viremia, indicative of a higher overall viral burden, is also hypothesized to be associated with severe dengue. Evidence for this has come from a number of studies examining dengue viremia in regard to disease severity and immune status, with the results generally indicating higher viremia in severe dengue and in secondary dengue infections, as compared to uncomplicated dengue and primary infections [37-39]. There is some inconsistency however, with other studies showing no association between viremia level and disease severity or immune status [40-43].

However, to date most of the studies have involved relatively small numbers of patients, and the numbers infected with individual serotypes were even smaller. Only a few studies have investigated dengue viremia in serial samples from large numbers of patients and considered timing during the illness evolution [39, 44]. Among the studies in Vietnamese children, higher dengue viremia was shown in the early acute phase in primary DENV1 infections, compared to secondary infections assessed at the same time-point [44, 45]. These studies also showed higher viremia in DENV1 than in DENV2 infections.

1.5.1.2 Virulence

Another viral factor contributing to dengue pathogenesis is viral virulence. All four serotypes can cause severe dengue. Some studies have suggested that DENV2 is more likely to be associated with severe dengue [37, 46-49], while in some other studies DENV3 was associated with outbreaks of severe dengue [50, 51]. Dengue severity was not different among DENV serotypes in other studies [52].

Among the various DENV2 viruses a number of different genotypes have been identified – in particular the American and the Asian genotypes - and there is evidence to support higher virulence associated with the Asian genotype [53]. With respect to DENV3 there are reports indicating that outbreaks of severe dengue were more likely to occur when there was emergence of a new clade of genotype [54, 55].

1.5.1.3 Role of non-structural protein1 (NS1) in pathogenesis

NS1 is a glycoprotein with a molecular weight of 46-55 kDa that exists in many different forms at different cellular locations [56]. It exists as a stable homodimer on the surface membrane of infected cells, and as a soluble hexamer in plasma. Soluble NS1 has been found in plasma from the first day of fever onset and lasting up to 14 days afterwards [57], and sNS1 levels in the acute phase have been found to correlate with dengue severity [58, 59].

The evidence from in-vitro studies suggests that NS1 and anti-NS1 antibodies may have important roles in dengue pathogenesis, especially in relation to plasma leakage, thrombocytopenia and coagulopathy. Thus sera from dengue patients, and purified antibodies against NS1, were found to act as anti-endothelial antibodies and induce apoptosis and cell lysis of endothelial cells via nitric oxide dependent pathways, thus leading to endothelial damage [60-62]. In addition, during the activation of endothelial cells, several inflammatory cytokines were also released, which, when combined with the endothelial apoptosis, may contribute to dengue vasculopathy [62]. Anti-NS1 antibodies were also found to cross-react with platelets and inhibit platelet aggregation [63, 64], potentially contributing to the thrombocytopenia seen in severe dengue. Similarly anti-NS1 antibodies and NS1 have been reported to cross react with several different molecules related to coagulation, such as human plasminogen and prothrombin/thrombin, thereby resulting in coagulation disorders [65-67].

Complement activation has also been suggested to contribute significantly to plasma leakage. Avirutnan et al (2006) found that both sNS1 and cell-associated NS1 could activate complement with the enhancement or support of anti-NS1 antibodies [68]. However, they also found that by binding to C4 or C4 binding proteins, NS1 attenuates classical and lectin pathway activation of complement and protects DENV from complement-dependent lysis [69, 70]. In addition, sNS1/anti-NS1 antibodies might facilitate dengue virus entry into a variety of cells [71, 72].

Table 1-1: Factors affect dengue pathogenesis

Factors and their roles in dengue pathogenesis	References
Viral factors	
Viremia Inconsistency in associations between viremia and dengue severity, as well as in associations between viremia and immune status - Higher viremia was associated with more DHF compared DF. - Higher viremia was found in DF than in DHF - There was no difference in viremia between DHF and DF - Higher viremia was found in secondary than in primary dengue infections - Higher viremia was found in primary than in secondary dengue infection - Viremia was not significantly different between primary and secondary dengue infections	Studies on humans - [37-39, 42] - [43] - [40, 59] - [42] - [40, 43-45] - [41]
Virulence - DENV2 was more likely to be associated with severe dengue - DENV3 was more likely to be associated with severe dengue - Secondary DENV2 infections with the Asian genotype were more likely to cause severe dengue than the American genotype. - Asian genotypes were better able to replicate in mosquitoes and human than American genotypes. - Outbreaks of severe dengue were associated with a genetic change in genotype III of serotype 3.	Studies on humans - [37, 47-49] - [50, 51] - [53] Studies on mosquitoes: [32, 73, 74] Phylogenetic studies: [54, 55]
NS1 - Higher levels of sNS1 in severe dengue (DHF and DSS) than in uncomplicated dengue (DF) - Sera from dengue patients and purified antibodies against NS1 were found to act as anti-endothelial antibodies, induce apoptosis, cell lysis and the expression and release of cytokines of endothelial cells - Anti-NS1 antibodies were found to react with platelets and inhibit aggregation. - NS1/anti-NS1antibodies were reactive with coagulation proteins - plasminogen and	Studies on humans: [58, 59] In vitro/animal models studies: - [60-62] - [63, 64] - [65-67]

prothrombin - NS1/anti-NS1 antibodies were found to activate complement as well as to attenuate complement activity - sNS1/anti-NS1 antibodies might facilitate dengue virus entry into a variety of cells	- [68-70] - [71, 72]
Immune factors	
Association of secondary dengue infection and severe dengue - Role of non-neutralizing antibodies against a previously encountered serotype (ADE hypothesis) - Antigenic sin or low immune reactive to current serotype than previous serotype	- In vitro study: [75] - Clinical studies: [76-78] - In vivo studies: [79, 80]
Cellular immunity - Activation of T cells and the release of cytokines found during the acute phase of dengue illness. DC and NK cells were also found to be activated in acute dengue	In vivo studies/review: [78, 80-84]
Cytokines - Increase in cytokines including IFN-y, TNF-α, IL-10, IL-6 and IL-8 in acute dengue	Studies on humans: [85-90]
Genetics factors - Differences in the following genes were found between severe and uncomplicated dengue: HLA, tumor necrosis factor alpha (TNF-α), transforming growth factor-beta 1 (TGF-β1), lymphotoxin alpha (LTA-α) and interleukin 10 (IL-10); FcγRII; vitamin D receptor; transporter associated with antigen processing (TAP) and human platelet antigen (HPA); Mannose binding lectin (MBL); JAK; MICB and PLCE1 - Differential gene expression of the following genes has been described in severe dengue compared to uncomplicated dengue: transcripts related neutrophil or innate immune response; genes related to type I interferon pathways, apoptosis, IL-6, NF-κB and IL-10 signaling pathways; genes related to T and NK lymphocyte response and anti-inflammatory reaction. Other studies have found associations of genes involved with metabolism, especially those related to mitochondrial ribosomal proteins and repair/remodeling, with severe dengue	- Studies of human gene genotyping/ genetic polymorphisms, or genome wide association studies using human samples: [91-105] - Gene expression studies using human samples: [59, 97, 106-109]

1.5.2 Host factors

The most severe manifestations of dengue occur when viremia has already peaked and begun to decrease. This suggests that the pathogenesis of severe dengue is at least partly related to host immune activity.

1.5.2.1 ADE and the potential role of immune status

Though the immunopathogenetic mechanisms underlying severe disease remain poorly understood, it is known that, following an initial infection with one dengue serotype, a second or subsequent infection with a different serotype frequently results in more severe disease rather than resulting in immunological protection from the previous infection. It is hypothesized that the binding of residual heterotypic non-neutralizing antibodies from the earlier infection with the new virus facilitates viral entry and replication, and in turn, the increased viral load causes an immunopathogenic cascade that alters microvascular function and results in a transient increase in vascular permeability that may be severe enough to cause dengue shock syndrome.

Halstead and O'Rourke suggested the role of non-neutralizing antibody in facilitating entry of dengue virus to host cells, and gave the first concept of antibody dependent enhancement (ADE) of viral infection [110]. In in-vitro experiments using peripheral blood mononuclear cells (PBMC) from people who were naïve to DENV (i.e. who had never been infected with dengue before), they showed that a small innoculum of virus, that was not able to infect the PBMCs alone, could infect the same cells when non-neutralizing dengue antibody was added to the culture medium. Higher viremia output was also seen in cell culture following supplementation with non-neutralizing antibody.

A lot of evidence from epidemiological studies supports the association of severe dengue and secondary infections. A prospective cohort study on about 2000 primary school children in Thailand from 1998 to 2000 showed an association between the presence of anti-dengue neutralizing antibodies to a previously encountered serotype with increased dengue severity [77]. In addition, studies on infants indicate that in the

initial months after birth maternal antibodies protect the infant from dengue, but that when the antibody levels decrease to low levels the risk for severe dengue increases [76]. Thus maternal antibodies transmitted to the baby prior to birth appear to function similarly to antibodies from a previous infection in older subjects, and a primary dengue infection occurring during the first year of life presents a clinical phenotype similar to a secondary infection in later life. A birth cohort study in Vietnam also confirmed the temporal association of infection enhancing activity of plasma from healthy children who had never had dengue before and age related case epidemiology during the first year of life [78]. The kinetics of the antibody response is also known to differ between primary and secondary dengue infections. In primary infections, IgM antibody develops rapidly, is detectable on day 3-5 of illness, reaches a peak two weeks later, and then declines to undetectable levels within 2-3 months. IgG appears later in the infection course, from the second week of illness onwards. By contrast in patients with secondary dengue, although the IgM time-course is similar the antibody levels achieved are thought to be significantly lower, while IgG appears in high titer either before or simultaneously with IgM [111]. In addition, IgG avidity is higher in secondary than primary infections [112].

It is important to note however, that although the ADE hypothesis helps to explain the association of secondary dengue infections and disease severity, it cannot explain the severe cases, including DSS, sometimes seen with primary infections [113].

1.5.2.2 Role of cellular immunology in dengue pathogenesis

Another hypothesis that has been proposed to explain the association of secondary heterotypic infection and severe dengue is the role of original antigenic sin. In fact this principle was first suggested by Halstead et al who showed that the highest neutralizing antibody titers in acute or late convalescent sera were to a prior viral serotype rather than to the current serotype [79]. However, Mongkolsapaya et al expanded on this idea, reporting that T cells likely play an important part in dengue pathogenesis based on this principle [80]. They found that in secondary infections, T cells have a relatively low affinity for the current serotype but have higher affinity for the serotypes from past infections. They hypothesized that T cells with low affinity to

the current serotype would be less effective at clearing the virus, thus potentially promoting immunopathology. However, this hypothesis is currently being challenged by work from Weiskopf et al, who found that T cell responses shifted from serotype-specific responses in primary infection toward conserved responses in secondary infection. In addition, no significant difference in the magnitude or avidity of T cell responses between serotype-specific and conserved responses was found [114].

T cells have been shown to be activated in the acute phase of dengue, with higher levels in severe dengue compared to dengue patients, and with the activation of CD8+ T cells being more profound than CD4+ T cell [81] [83]. As reviewed by Kurane et al, activated T cells are hypothesized to release cytokines, especially in more severe cases [83]. Increased levels of many different cytokines have been observed. Studies in Thai, Vietnamese and Indian children and infants all showed an increase in IFN-y, TNF-α in more severe dengue cases (DHF) compared to uncomplicated dengue (DF) or healthy patients [86, 88, 90]. Other studies reported increases in IL-10, IL-6 and IL-8 [85, 87, 89]. These inflammatory factors are hypothesized to mediate the increase of vascular permeability which leads to the severe complications of dengue, although the mechanism by which this occurs remains unclear.

In addition to T cells, other immune cells have been implicated in the host immune response against dengue, for example, dendritic cells (DC), antigen presenting cells and natural killer cells (NK cells). Both DC and NK cells were found to be activated in acute dengue and to release cytokines, which in turn are thought to contribute to enhanced endothelial permeability and plasma leakage [78, 82, 84, 115, 116].

1.5.2.3 Host genetics

A number of studies at both genome and transcription levels have been conducted to identify genetic factors associated with severe dengue. At the level of genome, both genotyping/polymorphism examination, and genome wide association studies have been used to identify alleles or genotypes associated with dengue/severe dengue. Human leukocyte antigens (HLA), which participate in host immune responses by presenting peptides to lymphocytes, have been investigated the most extensively, and associations both with severe dengue and with protection from severe disease have

been identified [92-94, 98, 100, 103, 105]. Associations with many other genes that encode for proteins related to host immune responses have also been examined and both susceptibility and protective relationships identified. Examples include the following: cytokines such as tumor necrosis factor alpha (TNF-α), transforming growth factor-beta 1 (TGF-β1), lymphotoxin alpha (LTA-α) and interleukin 10 (IL-10); FcγRII, which mediates viral entry in secondary dengue via antibody dependent enhancement; vitamin D receptor, an immune mediator expressed on monocytes and activated B and T cells; transporter associated with antigen processing (TAP) and human platelet antigen (HPA); mannose binding lectin (MBL), one of the first molecules acting in defense against infectious agents; and the JAK-type 1 interferon pathway [91, 95, 99, 101, 102, 104, 117]. A recent genome-wide association study involving 2000 Vietnamese children with DSS and 2000 controls found susceptibility loci for shock at MICB and PLCE1 [96].

Currently, microarray technology is being used extensively to investigate the pathogenesis of many infectious diseases including dengue at the level of transcription, i.e. gene expression. Though the results have not always been reproducible in different research studies, up-regulation of some genes related to host immune responses have been found in association with severe dengue: genes related to neutrophils or innate immune responses; type I interferon pathways, apoptosis, IL-6, NF-κB and IL-10 signaling pathways; T and NK lymphocyte response and anti-inflammatory reaction [59, 106-109]. Other studies have found associations with genes involved with metabolism, especially those related to mitochondrial ribosomal proteins and repair/remodeling associated with severe dengue [97, 109].

1.6 Diagnosis and clinical management

1.6.1 Clinical manifestations and routine laboratory findings

Following the bite of an infected mosquito there is a 3-7 day incubation period. The illness starts abruptly and follows 3 phases – the febrile phase, the critical phase and the recovery phase [118].

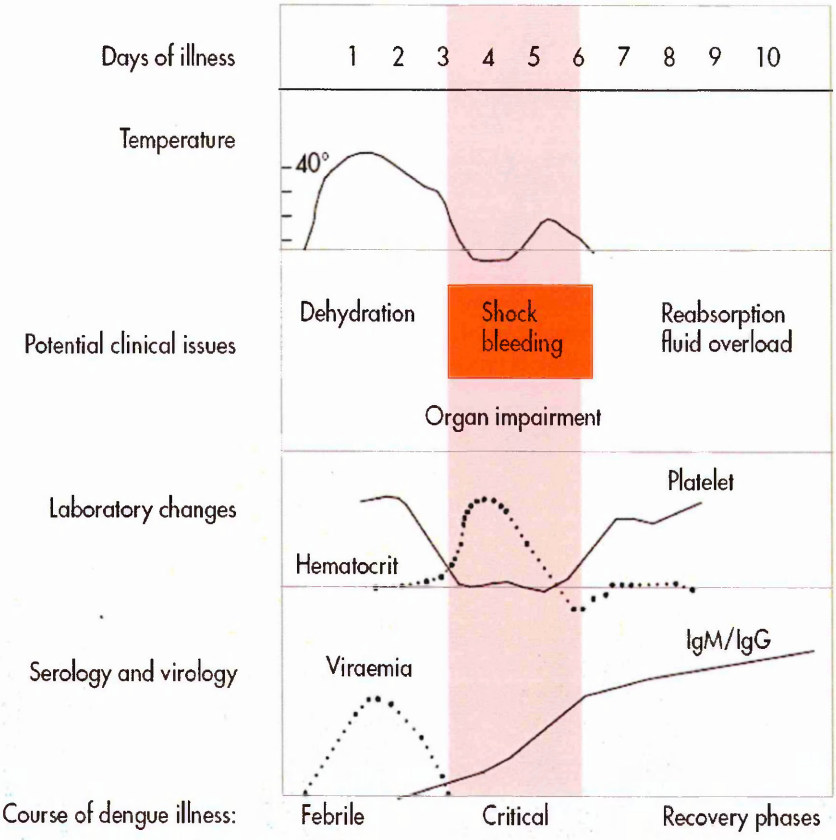
1.6.1.1 Febrile phase

The febrile phase starts with the abrupt onset of a high fever. Early in the course of illness dengue patients often present with facial flushing, skin erythema, body aches, myalgia, arthralgia, headache, anorexia, nausea and vomiting. These signs and symptoms also appear in other febrile illnesses (OFI) that are common in dengue-endemic regions and can therefore be confused with dengue, for example measles, typhoid, leptospirosis, and influenza. Other signs that are also commonly observed in dengue patients in this initial phase are mild hemorrhagic manifestations such as petechiae, bruising, nose or gum bleeding and a palpable liver. Laboratory findings include a decrease in white blood cell count and platelet count. This phase lasts for 2-7 days.

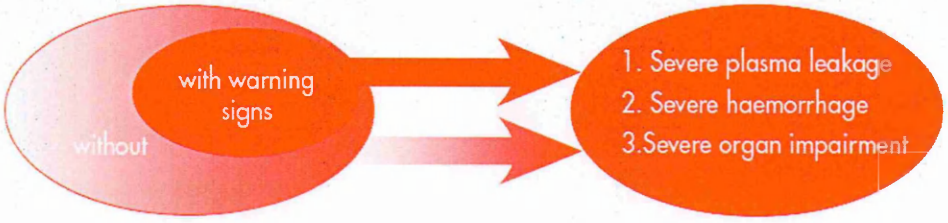
1.6.1.2 Critical phase

The critical phase occurs around the time of defervescence, usually between days 3–7 of illness, when the temperature drops to 37.5–38°C or less. While most patients start to improve at this time, a small proportion of people develop complications, in particular an unusual vasculopathy characterized by endothelial dysfunction and particularly affecting the microvasculature. The endothelial dysfunction results in increased vascular permeability and a plasma leakage syndrome that may be severe enough to cause hypovolaemic shock, and potentially death, if left untreated. There is some evidence to suggest that plasma leakage begins early in the dengue course, during the febrile phase. In a small proportion of cases, the plasma leakage is very profound with evidence of increased hemoconcentration, hypoproteinemia, pleural effusions and ascites. As leakage progresses compensatory mechanisms lead to a narrowing of the pulse pressure to maintain adequate circulation. When the pulse pressure narrows to less than 20 mm Hg, the degree of leakage becomes critical and the patient often develops tachycardia and cool, clammy skin; at this point DSS is diagnosed and the patient needs urgent fluid resuscitation. If shock is prolonged, poor organ perfusion may result in organ impairment, metabolic acidosis, and disseminated intravascular coagulation. Severe bleeding is sometimes associated with DSS, although usually bleeding is not severe even during the critical phase. During this

A



B



CRITERIA FOR DENGUE ± WARNING SIGNS

Probable dengue

live in / travel to dengue endemic area.
Fever and 2 of the following criteria:

- Nausea, vomiting
- Rash
- Aches and pains
- Tourniquet test positive
- Leukopenia
- Any warning sign

Laboratory-confirmed dengue

(important when no sign of plasma leakage)

Warning signs*

- Abdominal pain or tenderness
- Persistent vomiting
- Clinical fluid accumulation
- Mucosal bleed
- Lethargy, restlessness
- Liver enlargment >2 cm
- Laboratory: increase in HCT concurrent with rapid decrease in platelet count

*(requiring strict observation and medical intervention)

CRITERIA FOR SEVERE DENGUE

Severe plasma leakage

leading to:

- Shock (DSS)
- Fluid accumulation with respiratory distress

Severe bleeding

as evaluated by clinician

Severe organ involvement

- Liver: AST or ALT >=1000
- CNS: Impaired consciousness
- Heart and other organs

Figure 1-4: Dengue illness course and dengue classification

phase, coagulation abnormalities and thrombocytopenia are also commonly observed. Typically the platelet count begins to decrease during the febrile phase and reaches the nadir around day 5-6 of illness, i.e. during the critical phase.

1.6.1.3 Recovery phase

In the majority of patients the critical phase last for 48-72 hours, following which they enter the recovery phase. Laboratory abnormalities gradually return to the normal range and the clinical features improve. The three phases of dengue are illustrated in Figure 1-4A [1].

1.6.2 Clinical classification

As described above the clinical presentation of dengue varies widely. While the majority of patients infected with dengue recover after a short illness, a small proportion of patients progress to vasculopathy with increased vascular permeability, coagulation abnormalities, and bleeding manifestations. Previously, WHO classified symptomatic dengue into dengue fever (DF), and dengue haemorrhagic fever (DHF) grades I to IV, with grades III and IV alternatively called dengue shock syndrome (DSS). In 2009, because of some difficulties in application of this classification in practice, WHO issued a new guideline which classified symptomatic dengue into dengue with and without warning signs, and severe dengue (Figure 1-4B). Patients are classed as having severe dengue if they have any of the following criteria: severe plasma leakage leading to shock and/or fluid accumulation with respiratory distress; severe bleeding; and severe organ impairment. However, it has to be noted that even dengue patients without warning signs may develop severe dengue [1].

1.6.3 Laboratory diagnosis

A number of different laboratory techniques are currently used for dengue diagnosis. Each technique has advantages as well as disadvantages that together make it suitable for specific purposes.

1.6.3.1 Virus isolation

Serum, plasma or any biological specimens for viral culture should be collected early in the infection, during the period of viremia (2 to 3 days prior to, and up to 5 days after, the fever onset) [37]. Several systems have been developed for virus isolation, including mosquito systems, mouse brain inoculation, and culture in cell lines. Among them, mosquito inoculation is the most sensitive method. However, both mosquito and mouse brain inoculation are difficult and expensive techniques requiring particular safety facilities.

Cell lines are more widely used for dengue virus isolation. DENV is isolated by incubating the biological specimen with a suitable cell line, usually C6/36 cells (a mosquito cell line) or vero or BHK cells (mammalian cell lines). Infected cells are subsequently detected using DENV serotype-specific monoclonal antibodies by immunofluorescence assay. Common to all virus isolation methods, the sensitivity or isolation rate depends on the mosquito species or cell line chosen, the day of illness at sampling and the immune status of the patient. Viral culture is still the gold standard for dengue confirmation; however it is time consuming and has limited sensitivity, which makes it unsuitable for routine diagnosis.

1.6.3.2 Serological diagnosis

Serological tests target anti-DENV antibodies. IgM and IgG capture enzyme-linked immunosorbent assays (ELISA) (MAC and GAC) are widely used for dengue diagnosis. This is based on the principle that anti-dengue IgM and IgG antibodies increase in response to dengue infection. Although IgM can be detected as early as 3-5 days after illness onset, paired samples demonstrating seroconversion are required to make a definitive diagnosis. In addition, cross-reactions with other flaviviruses have been noted [119-121]. Epidemiological information about the prevalence of other flaviviruses should therefore also be included in the interpretation of serology results. Besides MAC and GAC, other serological methods such as anti-dengue IgG indirect ELISA, plaque reduction neutralization tests (PRNT), and haemaglutination inhibition (HI) are also used for dengue diagnosis. These tests are especially useful for

dengue sero-surveillance, and have been employed for differentiating between primary and secondary infections.

HI is based on the phenomenon that red blood cells will agglutinate in the presence of dengue antigen [1]. Antibodies in dengue patients' blood are able to neutralize the dengue antigens thus inhibiting this haemagglutination reaction. World Health Organization recommendations for using the HI test to differentiate between primary and secondary dengue requires paired samples with at least 7 days interval. In a primary infection, low levels of HI antibodies are seen in the first sample (S1). The titer then rises in the second sample but only to relatively low levels, usually lower than 1:1280. In contrast, the HI antibody titer rises rapidly in a secondary infection, usually exceeding the cut-off of 1:2560. However the HI technique is limited by several problems, in particular the test is time consuming to perform and different laboratories now use different cut-offs. Another disadvantage of HI is the fact that it requires paired samples, and in clinical settings the second sample is frequently not obtained as the patient has already recovered.

Recently, studies on dengue vaccines as well as evidence from sero-surveillance have indicated that levels of neutralizing antibodies as measured by PRNT correspond poorly to the level of protection against infection [122, 123]. However, PRNT are still considered to be a reliable method to detect previous exposure to different dengue serotypes, especially when performed in late convalescence, when the acute inflammatory response has subsided and cross-reactivity between serotype responses has decreased. Previous studies have demonstrated that in primary infections only antibodies against the DENV serotype that caused the recent infection are detected and measured in late convalescence, while in secondary dengue antibodies against several serotypes are present [124]. However, the test is cumbersome and time-consuming to perform and the sampling time-frame – several months after recovery – is not practical for clinical management. Even for research obtaining samples such a long time after recovery is challenging.

Because of the importance of knowing an individual's immune status in pathogenesis studies of natural human infections, and the complexity of HI or PRNT assays,

several groups have assessed alternative, more practical, methods to differentiate primary from secondary dengue infections. Most of these systems are based on knowledge of the expected antibody kinetics during dengue infections. Innis et al suggested using the IgM/IgG ratio with a cut-off of 1.78 [125]. Other authors also used IgM/IgG ratio but with different cut-offs, of 1.2 or 1.4 [126, 127]. In addition differences in absolute IgG levels and in avidity have been used by others to differentiate between primary and secondary infections [37, 127-129]. Widespread application of these methods is limited by the fact that most of the assays used were in-house assays, and in some studies the number of samples used for algorithm development was small. In addition, although levels of anti-dengue IgM and IgG clearly change during the acute illness, aside from the method presented by Cordeiro, none of the other methods take into account the evolving nature of the immune response over time.

1.6.3.3 DENV nucleic acid detection

Reverse transcriptase polymerase chain reaction (RT-PCR) can be used to detect the nucleic acid of DENV in a patient's blood during the viremic phase of dengue, up to 8 days after illness onset [130]. Various primer and probe sequences specific for DENV, or for a particular DENV serotype, have been developed for conventional RT-PCR and for real-time RT-PCR. These techniques now permit identification of the serotype, and quantification of the level of plasma viremia, with good sensitivity, specificity and limit of detection, especially for real-time RT-PCR. Multiplex real-time RT-PCR that allows detection of DENV serotypes in a single reaction has also been developed [131]. However, despite the overall advantages, the application of real-time PCR is limited by the requirement for expensive laboratory equipment and highly trained technicians.

1.6.3.4 Antigen detection

Currently a number of commercial tests have been developed for detection of the NS1 antigen; these have high specificity for DENV infection, and have been used

successfully for early diagnosis. However, the sensitivity varies depending on the DENV serotype, the immune status of the patient (primary or secondary infection), and the day of illness when the patient is assessed [132, 133].

1.6.4 Case management and disease prevention

Despite the increasing importance of dengue globally, current management guidelines continue to rely on supportive care and there are no specific therapies available for management of severe cases. The mainstay of treatment remains early recognition, regular monitoring and careful fluid management.

Several strategies are actively being investigated at the present time. Firstly there is considerable interest in the utility of antiviral reagents against dengue. An effective antiviral therapy could help a) to decrease the burden on healthcare services caused by high requirements for hospital admission in endemic areas (and by extension the economic burden on governments), and b) could help to reduce transmission of the virus to mosquitoes, thereby reduce ongoing transmission to new human hosts. There is increasing interest from industry in developing antiviral agents for dengue. These compounds target a number of possible virus and host proteins aiming to disrupt the virus life cycle by altering adhesion, RNA replication, viral assembly or other crucial events. Although many agents have been tested in-vitro (see review by Lim et al [134]), only a few have been assessed for efficacy and safety in formal clinical trials in humans. These include chloroquine, balapiravir (a polymerase inhibitor developed for treatment of the related virus, hepatitis C) and celgosivir (an iminosugar which participates in glycoprotein synthesis) [130, 135, 136]. Unfortunately, although the safety assessments were satisfactory, there was no evidence in these trials of a benefit in reducing plasma viremia or preventing the development of complications. Lovastatin, a drug primarily used to reduce cholesterol levels but which also has been reported to inhibit dengue virus replication in-vitro and to have endothelial stabilizing properties, is currently undergoing clinical testing for its efficacy and safety on dengue patients [137-139].

Another strategy that is being investigated involves suppression of the host immune response, which is hypothesized to contribute to the development of severe dengue, in

particular the endothelial dysfunction/vasculopathy. Prednisolone is a potent corticosteroid anti-inflammatory agent which is commonly used to treat diseases where the host immune response is thought to make a significant contribution to the severity of the disease, such as asthma [140, 141]. A number of clinical trials have examined the efficacy of corticosteroids in children with DSS, but with inconsistent results. However most of the work was carried out over 25 years ago, and the studies were small, underpowered, and lacked stringent randomization and clear allocation concealment [142-144]. Notably in these studies, the steroids were used after the onset of shock, which is now considered to be too late to be helpful. An alternative strategy that is currently being assessed is early use of corticosteroid therapy during the febrile phase in an attempt to prevent progression to severe disease. One randomized, blinded, placebo-controlled clinical trial of early prednisolone use was recently conducted in Vietnam, with the primary goal of assessing safety during the phase of active viral replication. The trial did not show evidence of harm with early prednisolone use compared to placebo, but also no evidence of prednisolone efficacy in preventing DSS. However it should be noted that the trial was not powered to an efficacy endpoint [36].

A number of vaccines for dengue are in development and several are undergoing clinical assessment [145, 146]. The most advanced is a recombinant tetravalent live-attenuated vaccine (CYD-TDV) developed by Sanofi-Pasteur. The vaccine comprises 4 recombinant live attenuated dengue viruses, expressed on a yellow fever virus 17D backbone. CYD-TDV has now been tested in phase 1, 2 and 3 clinical trials in thousands of healthy children from many countries, and has shown a good safety and immunogenicity profile (based on PRNT assays) [122, 147-153]. In the most recent report of the phase 3 trial involving 10,275 healthy children in Southeast Asia, the vaccine efficacy varied according to the serotype, being highest for DENV3 (78.4%) and lowest for DENV2 (35%), for an overall efficacy of 56.5% (95%CI 43.8 – 66.4%). The vaccine efficacy against severe dengue (DHF) and hospitalization were better, at 80% (95%CI 52.7 – 92.4) and 67.2% (95%CI 50.3 – 78.6) respectively [153].

1.7 Knowledge gaps and thesis objectives

Despite the efforts invested in studying dengue pathogenesis, the mechanisms underlying the severe disease manifestations remain poorly understood. Although it is not exclusive, a strong association between severe dengue and secondary infection is apparent, and in many research studies differentiation between primary and secondary infections is an important initial step to allow detailed exploration of mechanistic hypotheses. However, the methods currently in use are quite variable, often not practical, and usually do not take into account the evolving nature of the immune response over time. Therefore in the first part of my thesis (see Chapter 3) I set out to establish novel algorithms that permit differentiation of primary from secondary dengue infections at any day of illness with high accuracy, with the aim of applying the algorithm with the best performance to research investigating dengue viremia kinetics.

Viral burden is considered by many to be a major factor influencing disease severity and outcome in dengue, but existing knowledge of the magnitude and kinetics of plasma viremia (used as a surrogate measure of total viral burden) in association with different dengue serotypes, primary versus secondary immune status, and in relation to clinical disease severity, is limited and inconsistent. This is at least partly due to the small number of cases that have had detailed assessments in previous studies. In chapter 4, I report viremia kinetics in a variety of circumstances with serial samples from a large number of well characterized patients covering the full clinical disease spectrum.

As well as improving our understanding of the pathogenetic mechanisms underlying dengue. Knowledge of the expected magnitude and kinetics of viremia in different circumstances should prove invaluable for the design and conduct of therapeutic trials aimed at preventing and/or treating severe dengue. A randomized, placebo-controlled double-blind trial assessing the safety of early oral corticosteroid therapy in dengue patients was conducted at the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam between August 2009 and January 2011. In chapter 5, I focus on

investigating the effect of early prednisolone therapy on dengue viremia and on the whole blood transcriptional profile of patients enrolled in the study.

In summary, my work is aimed at investigating the following questions:

- Is it possible to develop algorithms to differentiate primary and secondary dengue infections based on serological responses using single specimens obtained on any day of illness during the acute phase with high accuracy?
- Are there measurable differences in DENV kinetics according to the infecting serotype and the host immune status? Specifically, does viremia kinetics differ between serotypes in patients with the same immune status? And is the viremia kinetics of the same DENV serotype actually different between primary and secondary infections? Finally is plasma viremia in the early days of illness independently associated with clinical outcomes from dengue?
- Although early therapy with prednisolone did not have a demonstrable clinical effect on patient outcomes in our recent study, did prednisolone influence viremia, antigenemia kinetics, immune responses or the whole blood transcriptional profile in the dengue patients? If so can this knowledge help us to understand more about dengue immunopathogenesis?

Chapter 2

MATERIALS AND METHODS

In this chapter, I will describe the laboratory techniques I used in my studies. Information on the clinical studies, patient enrolment, and clinical definitions, as well as the methodology for the statistical analyses, will be presented in detail later, in the relevant chapters.

2.1 RNA extraction

Supernatant from DENV1-4 viral culture in C6/36 cells and molecular water (Sigma) were used as external controls (positive and negative controls respectively) which were included whenever experiments were conducted. *Equine arteritis virus* (EAV) was added to all samples before extraction as an internal control to control the uniformity in all reactions.

QIAamp viral RNA mini kit (Qiagen) was used for the RNA extraction following the manufacturer's instructions. Firstly, 160µl of patient plasma, positive and negative controls, already mixed with 20µl EAV, was added into RNA free 1.5ml micro centrifuge tubes containing 560µl of prepared buffer AVL containing carrier RNA. The mixture was vortexed and incubated for 10 minutes at room temperature. In the next step, 560µl of ethanol was added to the tube, and then mixed by pulse vortex for 15 seconds. This was followed by a short spin to remove the drops from the lid. 600µl of the lysate was carefully transferred to QIAamp spin columns and then centrifuged at 8000 rounds per minute (rpm) for 1 minute. The spin column was transferred to another clean 2 ml collection tube. The same steps were repeated until all the lysate had been loaded to the column. Two washes were then performed, the first using 500µl of washing buffer of AW1 and the second using washing buffer of AW2. The RNA was eluted from the column with 60µl of elute buffer AVE for 10 minutes at room temperature and collected by centrifuging for 2 minutes at 14000 rpm. The RNA elute was kept at minus 80°C until used.

2.2 Two-step real-time multiplex reverse transcription (RT-) PCR

2.2.1 Procedure

Two-step real-time RT-PCR was performed as described elsewhere [154]. As the name indicates, the procedure includes two steps - firstly, reverse transcription for cDNA synthesis and then real-time PCR for quantification of the cDNA number in the samples.

2.2.1.1 cDNA synthesis

8µl RNA as extracted above was mixed with 5µl of Mix 1 (1µl of 100µg/ml random hexamers, 1µl of 10mM dNTP and 3µl molecular water) and incubated at 65°C for 5 minutes. After the incubation, the reaction was transferred immediately on ice, where 7µl of Mix 2 (4µl of 5X buffer, 1µl DTT, 0.4µl of 40U/µl RNase inhibitor, 0.2µl of 200U/µl SuperScript III Reverse Transcriptase and 1.4µl molecular water) was added. The reaction was incubated at 50°C for 60 minutes then 72°C for 5 minutes. cDNA product was kept at -20°C until used.

2.2.1.2 Real-time PCR

Each PCR reaction only detects and quantifies one DENV serotype. The sequences of specific primers and probes for DENV serotypes and EAV were listed in Table 2-1. A linearized plasmid containing the cloned target amplicon, which was diluted in 10 fold series, was used as standard for the real-time RT-PCR reaction. Firstly, the PCR master mix was prepared as described in Table 2-2, then 4 µl of cDNA of the patients' samples, positive, negative controls or standard DNA was added to the reaction. PCR reactions were run on thermo cycler (Biorad) as follows: 95°C for 15 minutes; and 45 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds and fluorescent measurement.

2.2.2 Controls and standards

Results of an experiment were accepted if positive and negative controls gave correct results. In addition, the Ct values of the internal controls of all samples had to be in the allowable range. In cases where some values were out of the allowable range, the results would be accepted if the criteria in Table 2-3 were met.

Table 2-1: Dengue and EAV specific primers and probes for two-step real-time RT-PCR

Primer/Probe	Primer/Probe Sequences	Position	Product size in base
DENV1 - Forward	ATCCATGCCCACCAYCAATG	9960 - 9980	162
DENV1 - Reverse	CAGGGATCCACACCAYTGATC	10100 - 10121	
DENV2 - Forward	ACAAGTCGAACAACCTGGTCC AT	9938 - 9941	178
DENV2 - Reverse	GCCGCACCATTGGTCTTCTC	10095 - 10115	
DENV3 - Forward	TTTCTGCTCCCACCACTTTCAT	9719 - 9741	216
DENV3 - Reverse	TGGCGTTGGATGCYAGTCT	9915 - 9934	
DENV4 - Forward	GYGTGGTGAAGCCYCTRGAT	9587 - 9607	178
DENV4 - Reverse	AGTGARCGGCCATCCTTCAT	9744 - 9764	
EAV- Forward	CATCTCTTGCTTTGCTCCTTAG	1847 - 1868	133
EAV- Reverse	AGCCGCACCTTCACATTG	1963 - 1980	
DENV1-Probe	5'(FAM)TCAGTGTGGAATAGGGTTTGGATAGAGGAA3'(TAMRA)		
DENV2-Probe	5' FAM GTT+T+Tg+T+CT+TC+CA+TCCA3'BHQ-1 (+ indicates *LNAbase*)		
DENV3-Probe	5' (FAM)AAGAAAGTTGGTAGTTCCCTGCAGACCCCA3'(TAMRA)		
DENV4-Probe	5' (FAM)ACTTCCCTCCTCTTYTTGAACGACATGGGA3'(TAMRA)		
EAV - Probe	Cy5CGCGCTCGCTGTCAGAACAACATTATTGCCCACAGCGCG3'(BHQ3)		

Y stands for C or T nucleotide, R for A or G.

The standard curves which were generated automatically by the Opticon software were accepted if R coefficient ≥ 0.9.

Table 2-2: Two-step real-time RT-PCR master mix components

Reagents	Working concentration	µl/reaction
DENV1 (or DENV2, 3, 4) Forward primer	10µM	1.5
DENV1 (or DENV2, 3, 4) Reverse primer	10µM	1.5
EAV Forward primer	10µM	1.5
EAV Reverse primer	10µM	1.5
DENV1 (or 2, 3, 4) probe	1µM	5
EAV probe	5µM	1.5
$MgCl_2$	25mM	2.5
10X HotstarTaq buffer	10X	2.5
dNTPs	10mM	1
HotstarTaq	5U/µl	0.2
Molecular water		2.3
Total		**21**

Table 2-3: Interpretation of two-step real-time RT-PCR results

EAV/DENV	Result			
	(+/+)	(+/-)	(-/+)	(-/-)
	Pass	Pass	Pass	Fail

2.2.3 Interpretation

The two-step real-time RT-PCR results were collected and analyzed using Opticon software (Biorad). The units of the RT-PCR are presented as copies/ml. Patient samples were defined as negative if the Ct value was higher than 40. The limit of detection (LOD) for each serotype was about 5,000copies/ml of plasma.

2.3 One-step real-time multiplex reverse transcription (RT-) PCR

2.3.1 Procedure

The procedure for one-step real-time multiplex RT-PCR has been described elsewhere [131]. There are two sets of multiplex assays to detect i) DENV1, DENV3 and EAV or ii) DENV2, DENV4 and EAV simultaneously. Sequences of specific probes and primers for each DENV serotype and EAV are described in Table 2-4. A linearized plasmid containing the cloned target amplicon, which was diluted in 10 fold series, was used as standard for the real-time RT-PCR reaction.

Firstly, the PCR master mix was prepared as described in Table 2-5, then 6µl of RNA (which had been previously extracted from the patient's plasma), positive or negative controls, or standard DNA was added to the reaction. PCR reactions were run on a LightCycler II PCR machine (Roche) as follows: 61°C for 10 minutes; 95°C for 2 minutes; and 45 cycles of 95°C for 15 seconds, 60°C for 30 seconds and fluorescent measurement. Signals were read at wavelength 498-580, 440-488 and 618-660 for DENV1/DENV2, DENV3/DENV4 and EAV respectively.

2.3.2 Controls and standards

Controls and standards for the one-step real-time multiplex RT-PCR were similar to the two-step real-time RT-PCR described above. In addition, the standard curves which were generated automatically by the **LightCycler software** were accepted if efficiency >1.7 and error <0.2.

Table 2-4: Dengue and EAV specific primers and probes for one-step real-time multiplex RT-PCR

Primer/Probe	Primer/Probe sequences (5'-3')	Position	Product size in base
DENV1- Forward	ATCCATGCCCAYCACCAAT	9865 - 9883	100
DENV1- Reverse	TGTGGGTTTTGTCCTCCATC	9945 - 9964	
DENV2- Forward	TCCATACACGCCAAACATGAA	9859 - 9879	125
DENV2- Reverse	GGGATTTCCTCCCATGATTCC	9963 - 9983	
DENV3- Forward	TTTCTGCTCCCACCACTTTC	9591 - 9610	118
DENV3- Reverse	CCATCCYGCTCCTTGAGA	9691 - 9708	
DENV4- Forward	GYGTGGTGAAGCCYCTRGAT	9587 - 9607	178
DENV4- Reverse	AGTGARCGGCCATCCTTCAT	9744 - 9764	
EAV- Forward	CATCTCTTGCTTTGCTCCTTAG	1847 - 1868	133
EAV- Reverse	AGCCGCACCTTCACATTG	1963 - 1980	
DENV1-Probe	5' (FAM) TCAGTGTGGAATAGGGTTTGGATAGAGGAA 3'(BHQ1)		
DENV2-Probe	5'(FAM) AGGGTGTGGATTCGAGAAAACCCATGG 3'(BHQ1)		
DENV3-Probe	5'(Cyan500) AAGAAAGTTGGTAGTTCCCTGCAGACCCCA 3'(BHQ1)		
DENV4-Probe	5'(Cyan500)TTCCCTCCTCTTYTTGAACGACATGGGAAAGGT G 3'(BHQ1)		
EAV - Probe	5'(Cy5)CGCGCTCGCTGTCAGAACAACATTATTGCCCACAG CGCG 3'(BHQ3)		

Y stands for C or T nucleotide, R for A or G.

Table 2-5: One-step real-time multiplex RT-PCR master mix components

Reagents	Working concentration	µl/reaction
Mix of DENV1 (or DENV2) Fw – Rev primers	20µM	1
DENV1 (or DENV2) probe	10µM	0.28
Mix of DENV3 (or DENV4) Fw – Rev primers	20µM	1
DENV3 (or DENV4) probe	10µM	0.28
Mix of EAV Fw - Rev primers	20µM	0.2
EAV probe	10µM	0.08
Activator		1.4
Enhancer	20X	1
RNA master mix	2.7	7.4
H2O		1.36
Total		**14**

2.3.3 Interpretation

The one-step real-time multiplex RT-PCR results were collected and analyzed using the LightCycler® 480 SW 1.5, the software provided with Lightcycler II PCR machine (Roche). The units of the RT-PCR are presented as copies/ml. The limit of detection (LOD) for each serotype was identified via a rigorous validation procedure, and is presented in the Table 2-6 [131]. Viremia levels that were lower than the LOD were considered to be negative.

Table 2-6: Limit of detection of one-step real-time multiplex RT-PCR assay

	DENV1	DENV2	DENV3	DENV4
LOD (copies/reaction)	5	1	5	10
LOD (copies/ml plasma)	300	60	300	600

2.4 Capture IgM/IgG enzyme-linked immunosorbent assay (ELISA)

2.4.1 Controls

Acute plasma from Vietnamese confirmed dengue patients which gave strong positive results with the capture IgM/IgG ELISA were pooled to create the positive control. The negative control sample was a mixture of plasma from dengue-naïve healthy adult volunteers who had just arrived from regions without known dengue transmission and who had negative results on the Panbio IgG indirect ELISA.

2.4.2 Procedure

The procedure for IgM/IgG has been described elsewhere [132, 155]. Briefly, 96 well plates (Maxisorp, Nunc) were coated with 100µl/well of anti-human IgM (A0425, Dako) or anti-human IgG (I2136, Sigma) at a dilution of 1:2000 in 0.05 M carbonate-bicarbonate buffer, pH9.6 (C3041, Sigma) overnight at 4°C. After 3 washings with phosphate buffer saline–TWEEN (PBST), each well was blocked with 200µl of 3% bovine serum albumin (BSA, A7906, Sigma) in phosphate buffer saline (PBS) for 2 hours at room temperature. Next, the plate was washed again and then incubated for 2 hours at room temperature with 100µl/well positive, negative controls or samples which were diluted 1:100 in 0.1% BSA-PBS. After washing, the assay was continued by adding 100µl of pooled DENV1-4 antigen (pooled C6/36 cultures of DENV1-4) to each well and incubating at 4°C overnight. Afterwards, the plate was washed and incubated with a cocktail of mouse monoclonal anti-DENV1-4 E protein antibodies (100µl/well) for 1 hour at room temperature. The antigen-antibody complex was detected with 100µl of 1:2000 diluted anti-mouse Ig Horseradish Peroxidase (HRP) (P260, Dako) for 1 hour in the dark at room temperature. The color reaction was developed with substrate o-phenylenediamine dihydrochloride (OPD, Sigma) in the dark for 30 minutes and then stopped by adding 50µl of 10% H_2SO_4. The optical density (OD) was read at 490nm with Microplate Reader and analyzed with Microplate Manager software (Biorad).

2.4.3 Calculation and interpretation

All of the OD values of negative and positive controls and samples were normalized by subtracting to the mean background absorbance value (ODB) which was the mean OD of the blank wells where the sample and the DENV antigen was PBS. Negative and positive control means (NCM and PCM) were the mean of corresponding controls. The assay cut-off (CO) was defined as 5 times the NCM. Sample ratio (RS) was calculated by taking the normalized sample OD (ODS) minus the mean of background (ODB) and dividing by the assay cut-off: RS=(ODS-ODB)/CO. The result was interpreted as follows:

- If RS <0.8, result was interpreted as negative
- If RS >1.2, result was interpreted as positive
- If $0.8 \leq RS \leq 1.2$, result was interpreted as equivocal

2.5 Anti-E protein IgG indirect ELISA

2.5.1 Controls

Acute plasma from Vietnamese confirmed dengue patients which gave strong positive results with the Panbio IgG indirect ELISA were pooled to create the positive control. The negative control sample was a mixture of plasma from dengue-naïve healthy adult volunteers who had just come from regions without known dengue transmission and had negative results with Panbio IgG indirect ELISA.

2.5.2 Procedure

Firstly, 48 wells of 96 well plates (Maxisorp, Nunc) were coated with 50µl/well of a cocktail of four recombinant DENV1-4 E proteins (Hawaii Biotech) at a concentration of 0.5µg/each serotype/ml in carbonate and bicarbonate buffer (C3041, Sigma) at 4°C, overnight. The remaining wells were uncoated. After washing 3 times the plates were blocked with 200µl/well of 3% bovine serum albumin (BSA, A 7906, Sigma) in PBS for 1 hour at room temperature. After washing again, 100µl of 1:100 diluted plasma, positive control or negative control was added to the coated and

uncoated wells and incubated for 1 hour at room temperature. The assay was continued with washing and then adding 100µl/well of goat anti-human IgG peroxidase conjugate. 100µl of TMB was used as the substrate for color reaction for 10 minutes in the dark at room temperature. The optical densities of the wells were measured at 450nm.

2.5.3 Calculation and interpretation

All of the OD values of negative, positive controls and samples were normalized by dividing by their own background absorbance value which was OD of uncoated wells. Negative and positive control means (NCM and PCM) were the mean of corresponding controls. The assay cut-off (CO) was defined as 5 times of NCM. Sample ratio (RS) was calculated by taking the normalized sample OD dividing by the assay cut-off. The result was interpreted as follows:

- If RS <0.8, result was interpreted as negative
- If RS >1.2, result was interpreted as positive
- If $0.8 \leq RS \leq 1.2$, result was interpreted as equivocal

2.6 Panbio anti-dengue IgG indirect ELISA

2.6.1 Procedure

The Panbio anti-dengue IgG indirect ELISA was performed following the instructions of the manufacturer. Briefly, samples, negative controls, positive controls and calibrators were firstly diluted 1:100 with serum diluents and then added to the 96 well-plate (100µl/well), which had been coated with dengue antigens by the manufacturer. The plate was incubated at 37°C for 30minutes. After washing 6 times with diluted washing buffer, to each well was added 100µl of horse radish peroxidase (HRP) conjugated anti-human IgG. The plate was incubated for another 30 minutes at 37°C. After another wash, 100µl of TMB was added to each well and incubated for 10 minutes in the dark at room temperature. The reactions were stopped with 100µl of stop solution. The optical densities were measured at 450nm with reference filter of 600-650nm.

2.6.2 Calculation and interpretation

The cut-off value was a multiplication of the calibrator factor and the average value of absorbance of the 3 calibrators. The index value was calculated by dividing the absorbance of the samples by the cut-off value, with the Panbio unit taken as 10 times the index value. The interpretation of results was as follows:

- If index value < 0.9, result was interpreted as negative
- If index value > 1.1, result was interpreted as positive
- If 0.9 < index value <1.1, result was interpreted as equivocal

2.7 Qualitative NS1 ELISA

2.7.1 Procedure

The Platelia^{TM} Dengue NS1 Ag Kit (BIO-RAD) was used for qualitative sNS1 assays and the procedure was as per the manufacturer's instructions. Briefly, 50µl of diluent and 50µl of plasma or control solution (negative or positive) or calibrator (cut-off) and 100µl of diluted conjugate were contributed to the plate. The reaction was incubated at 37°C for 90 minutes. After washing, 160µl of chromogen solution was added into each well and incubated for 30 minutes in a dark environment at room temperature. The color reaction was stopped using 100µl/well of 1N sulfuric acid solution. The optical density was read at 450/620 nm.

2.7.2 Calculation and interpretation

The sample ratio (RS) was calculated by dividing the optical density (OD) of the sample by the OD of the cut-off. The results of the assay were acceptable if the cut-off OD > 0.2, and the RS of negative and positive controls was <0.4 and >1.5 respectively. The result interpretation was as follows:

- If RS <0.5 - result was interpreted as negative
- If RS >1.0 - result was interpreted as positive
- If $0.5 \leq RS \leq 1.0$ - result was interpreted as equivocal

2.8 Algorithm for laboratory confirmation of dengue [132]

A case was considered to have confirmed dengue based on the serology, PCR and NS1 results, using an algorithm previously established in our laboratory [132]. For serological diagnosis, two samples were required. The first samples (S1) were collected when patients first presented at the health facility, usually within 72 hours of fever onset. The second samples (S2) were collected on day 6 or more of illness, with a gap of at least 3 days between the two samples. Patients were confirmed as having acute dengue if any of the following conditions were satisfied: i) positive with one of 4 serotypes of DENV in PCR test or positive with NS1 test, or ii) IgM seroconversion in MAC ELISA from S1 to S2, or iii) IgM positive in both S1 and S2 with IgG conversion from S1 to S2 or iv) IgM positive in both S1 and S2 with a 20% rise in IgM in S2 (S2 IgM $\geq$ 1.2 X S1 IgM units). Patients were confirmed negative for dengue infection if they had negative PCR for all 4 serotypes, were negative for NS1 and had negative IgM and IgG serology results in both S1 and S2. Other conditions were defined as recent DENV infection (IgM positive in a single specimen, or in paired samples but with no significant (< 20% rise in units), or acute flavivirus infection (IgG seroconversion from S1 to S2), or recent flavivirus infection (IgG positive in S1 and S2), or indeterminate if none of the above conditions were met.

2.9 Plaque reduction neutralization test (PRNT)

The PRNT assay was conducted at Laboratory of Infectious Diseases (NIAID, NIH, USA) of Professor Steve Whitehead as described elsewhere [156, 157]. Strains of DENV1 Puerto Rico/94, DENV2 NGC prototype, DENV3 Sleman/78, DENV4 814669 were used for the PRNT assay. Briefly, plasma was heat inactivated at 56°C for 30 minutes. Next, a serial of 2-fold dilutions beginning at 1:10 were prepared in serum-free medium containing 0.25% human serum albumin. The virus which was previously diluted to 500 plaque-forming units/ml in medium containing guinea pig complement (Cambrex) at a complement fixation titer of 1:10 was added to equal volumes of diluted plasma and incubated at 37°C for 30 min. 0.1 ml of virus-serum mixture was incubated with monolayer cultures of Vero cells on 24-well plates for 60

min at 37° C. After the incubation, the wells were overlaid with medium containing 1% methylcellulose and 2% fetal bovine serum and were incubated at 37°C for 4–5 days. The plaques were visualized by immunoperoxidase staining, and a 60% PRNT ($PRNT_{60}$) titer was calculated. The limit of detection was a titer of 10.

2.10 Cytokine quantification

IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IFNγ, TNFα and IP10 were quantified using Bio-Plex Precision Pro Assays, Human cytokine 10-Plex and Human IP10 (Biorad).

2.10.1 Procedure

The procedures were carried out according to the manufacturer's instructions. Briefly, after pre-wetting the 96-well filter plate with 200µl of assay buffer, 50µl of the beads was added to each well and vacuum filtered. After washing, 50µl of standard control or sample was added to each well and incubated for 1 hour in the dark at room temperature. The assay was continued with vacuum filtering, washing, and incubating with 25µl 1X detection antibody at room temperature in the dark for 30 minutes. After filtering and washing, the bead-cytokine-antibody complex was detected by incubating with 50µl of 1X streptavidin-PE for 10 minutes in the dark at room temperature. After filtering and washing, 125µl of assay buffer was added to each well and the assay plate was shaken for 30 seconds at 1,100 rpm before acquiring data using a multiplex array reader from Luminex Systems (Bio-Plex 200 system from Bio-Rad Laboratories).

2.10.2 Calculation and interpretation

The data were analyzed and the concentration of each cytokine was calculated using Bio-Plex Manager Software. The units for the cytokine levels were pg/ml. The limits of detection were 0.23 (IL-1β), 0.84 (IL-2), 0.14 (IL-4), 1.5 (IL-5), 1.23 (IL-6), 0.96 (IL-10), 0.2 (IL-12p70), 1.19 (IL-13), 0.34 (IFNγ), 0.14 (TNFα) and 10 (IP10) (all pg/ml).

2.11 T cell phenotyping

150µl whole blood was mixed with 5µl of antibody cocktail of CD3-APC (BD – 555342, an antibody against T cells in general), CD4-APC-Cy7 (BD – 341105, an antibody against $CD4^+$ T cells), CD8-P (BD – 555367, an antibody against $CD8^+$ T cells), CD38-PE-Cy7 (BD – 335808, an antibody against activated T cells) and HLA-DR-PerCP (BD – 347364, an antibody against activated T cells) in BD Falcon tubes for 10 seconds. This mixture was then incubated at room temperature in the dark for 20 minutes. 1.5 ml FACS lysing buffer (BD - 349202) was added to the mixture and mixed for 10 seconds, then incubated at room temperature in the dark for 10 minutes. The assay was continued with centrifugation at 1600 rpm for 5 minutes. The supernatant was discarded and the pellet was washed with 2ml of washing buffer (3% heat inactivated FCS (Sigma) and 1mM EDTA (Merck) in PBS) and resuspended in 500µl of FACS Perm2 (BD - 340973). This suspension was well mixed for 10 seconds and then incubated for 10 minutes at room temperature in a dark environment. Centrifugation at 1600 rpm for 5 minutes was performed to collect the pellet which was subsequently washed with 2ml of washing buffer and incubated with intracellular antibody against Ki67-FITC (BD - 51-36524X, an antibody against activated T cells) for 15 minutes at room temperature in dark. After washing, 150µl of 1% Paraformaldehyde (Merck) solution was added to the stained cells for fixation. Data were acquired using a FACS Canto flow cytometer (Becton Dickinson) and the raw data were analyzed using Cellquest or FlowJo software.

2.12 Expression microarrays

2.12.1 Whole RNA extraction

2.5ml of whole blood which had been stored in Paxgene RNA tubes (Qiagen) was used for RNA extraction using Paxgene RNA kits (Qiagen) according to the manufacturer's instructions. In brief, the whole blood was transferred to a 50ml Falcon and centrifuged at 3000g for 10 minutes to collect the pellet. After washing with 5ml RNase-free water, the pellet was lysed by adding 350µl of BR1 and 300µl of BR2 and 40µl proteinase K respectively and incubating on a shaker at 55°C for 10

minutes. The lysate was then transferred into a PAXgene shredder spin column placed in a 2ml processing tube and centrifuged for 3 minutes at maximum speed. The entire supernatant of the flow-through fraction was carefully transferred to a fresh 1.5 ml centrifuge tube without disturbing the pellet in the processing tube. 350µl of ethanol (96-100%) was added and mixed with the solution and then transferred immediately into a PAXgene RNA spin column placed in a 2ml processing tube which was then centrifuged for 1 minute at 10,000 g and washed with 350µl of BR3. 80µl of DNase I incubation mix (10µl of DNase I stock solution and 70µl Buffer RDD) was directly pipetted onto the PAXgene RNA spin column membrane and incubated for 15 minutes at room temperature. In the next step, 350µl of BR3 was continued to add to the column which was then centrifuged for 1 minute at 10,000g. The column was washed twice with 500µl of BR4. RNA was eluted from the column by 2 further incubations of 40µl of BR5 for 2 minutes with centrifugation for 1 minute at 10,000g. The RNA was quantified and qualified using Nanodrop. RNA was kept at minus 80°C until use.

The Illumina® TotalPrep™-96 RNA Amplification Kit (Ambion) was used for cRNA synthesis and purification according to the manufacturer's instructions, including the following steps (from 2.12.2 - 2.12.4).

2.12.2 Reverse transcription to synthesize first strand cDNA

11µl of RNA suspension containing about 500ng of RNA was mixed with 9µl of reverse transcription master mix (2µl 10X first strand buffer, 4µl of dNTP mix, 1µl of T7 oligo(dT) primer, 1µl of RNase inhibitor and 1µl of ArrayScript). The reaction was incubated for 2 hours at 42°C. After incubation the tube was centrifuged briefly to collect the reaction product at the bottom of the tube.

2.12.3 Second strand cDNA synthesis and purification

The second strand master mix (63µl nuclease-free water, 10µl 10X second strand buffer, 4µl dNTP mix, 2µl DNA polymerase and 1µl RNase H) was prepared on ice and mixed with product from the first strand cDNA synthesis. The reaction was incubated at 16°C for 2 hours and then put on ice before cDNA purification.

180µl of resuspended cDNAPure was added to each second strand cDNA synthesis reaction. The mixture was transferred to a U-Bottom Plate and gently shaken for 2 minutes to thoroughly mix the sample with the cDNAPure. The plate was put onto a magnetic stand for about 5 minutes so that the magnetic beads could be captured. When the capture was complete, the supernatant was carefully aspirated without disturbing the magnetic beads. The plate was removed from the magnetic stand and the beads were washed twice with 150µl of cDNA wash buffer and then dried by shaking vigorously for 2 min. The cDNA was eluted from the beads by incubating the beads with 20µl preheated 55°C nuclease-free water and vigorously shaking for 3 minutes. cDNA was collected by putting the plate on a magnetic stand for 5 minutes and carefully transferring 17.5µl of the supernatant into the wells of a new PCR plate.

2.12.4 In vitro transcription (IVT) to synthesize anti-sense RNA and purification

7.5µl of IVT master mix (2.5ul Biotin-NTP mix, 2.5µl T7 10X reaction buffer, 2.5µl T7 enzyme mix) was added and gently mixed with each sample (purified cDNA). The reaction was incubated at 4°C in the dark overnight. Each tube of cRNA product was mixed with 70µl cRNA Binding Mix and 95µl of absolute ethanol. The mixture was transferred to a U-Bottom Plate and shaken vigorously for 2 minutes. The plate was put on the magnetic stand for about 5 minutes until the magnetic beads were completely captured, then the supernatant was carefully aspirated without disturbing the magnetic beads. The plate was removed from the magnetic stand and the magnetic beads were washed twice with 100µl of cRNA Wash Solution. After the magnetic beads had been dried by vigorously by shaking the plate for 1 minute, purified cRNA was eluted by adding 40–100µl of preheated (55°C) cRNA Elution Buffer to each sample and shaking vigorously for 3 minutes. The supernatant containing cRNA was collected and transferred to a nuclease-free PCR plate, then kept at –80°C until use.

2.12.5 Array hybridization

The Direct Hybridization assay in this step was carried out as the instructions from the manufacturer. Briefly, for each sample 2µg of cRNA in 5µl of RNase-free water was heated at 65°C for 5 minutes and cooled at room temperature before being mixed with 10µl of Hyb which had been previously heated at 58°C for 10 minutes. The samples

were loaded onto the BeadChip (Human HT-12 v4.0). The Hyb Chamber Insert containing sample-laden BeadChips was loaded into each Hyb Chamber, which was incubated in a preheated oven at 55°C for 20 hours. The assay was continued with these washing steps; the chip was washed firstly in 1X High-Temp Wash buffer which was preheated at 55°C in Hybex Waterbath for 10 minutes, and then in Wash E1BC solution for about 5 minutes on an orbital shaker. The rack containing the BeadChip was transferred to a staining dish containing absolute Ethanol and incubated there for 10 minutes on the orbital shaker. It was then returned to the Wash E1BC solution for another 2 minutes on the orbital shaker. After the washing, the BeadChip was blocked with 4ml Block E1 buffer for 10 minutes and stained with 2ml of streptavidine-Cy3 in Block E1 buffer for 10 minutes and then washed with Wash E1BC for 5 minutes. The BeadChip was dried by centrifugation at 1,400 rpm for 4 minutes. The BeadChip was scanned on the Illumina BeadArray Reader. From there the gene expression data were extracted.

2.13 Quantitative RT-PCR (qRT-PCR) validation

2.13.1 Reverse transcription

cDNA was synthesized from RNA using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) following the manufacturer's instructions. First 500ng of RNA in 10µl of RNase-free water was added to 10µl of 2X-reverse transcription master mix. The reaction was loaded to the thermal cycler and incubated at 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes.

2.13.2 Pre-amplification

Taqman assays which are the sets of primers and probes for the 31 genes of interest, and 18S as a house keeping gene, were prepared by mixing them together with equal ratio. 1.25µl of cDNA was added to 3.75µl of master mix for pre-amplification (2.5µl of Taqman Preamp master mix (2X) and 1.25µl of pooled assay mix (0.2X)). The reaction was loaded to the thermal cycler and run under the following conditions: 95°C for 10 minutes and then 14 cycles of 95°C for 15 seconds and 60°C for 4 minutes.

2.13.3 Amplification

The 48.48 Dynamic Array^{TM} FC (Fluidigm) was used for the realtime RT-PCR following the instructions of the manufacturer. First the fluidigm was primed with control line fluid in an Integrated Fluidic Circuit (IFC) Controller MX machine. Next, the 10x Taqman assays (5µl: 2.5µl of 20X Taqman Gene Expression and 2.5µl of 2X Assay Loading Reagent) and mixture of sample and amplification premix (2.5µl of Taqman Universal PCR Master Mix, 0.25µl of DA Sample loading reagent, 2.25µl of 1:5 diluted Pre-amplification product) were added to the chips. The micro fluidic chip was then run in the BioMark instrument (BioMark). PCR results were collected and verified using the BioMark Data Collection software and the BioMark Real-Time PCR Analysis software.

Chapter 3

DEVELOPING ALGORITHMS TO DIFFERENTIATE PRIMARY FROM SECONDARY DENGUE VIRUS INFECTIONS

3.1 Introduction

As discussed in the introductory chapter, immune status is considered to be an important factor contributing to dengue pathogenesis. Currently, a number of methods are used to differentiate primary from secondary dengue. Previously HI was recommended as the gold standard for this purpose; however several relatively complicated preparatory steps are needed and the test is time-consuming to conduct. Also HI fails to discriminate between infections by closely related flaviviruses, such as Japanese encephalitis virus (JEV). Another disadvantage is that paired samples are required for a reliable result – i.e. acute and convalescent samples – and in clinical practice the second sample is frequently not obtained as the patient has already recovered. PRNT is another method used to define dengue immune status. Although rather cumbersome and time consuming to perform, the neutralization assay is considered to be the serological technique with the greatest specificity, allowing differentiation from other flaviviruses as well as determination of neutralizing antibody levels to the four dengue serotypes. However, since the specificity of neutralizing antibodies increases over time, samples for PRNT are best collected in late convalescence in order to avoid cross-reactive responses, i.e. several months or years after recovery. This is not practical or useful in the clinical context, and it can also be a problem in research studies, as it can be difficult to keep track of study participants especially in dengue endemic areas.

In comparison to both HI and PRNT, serological testing using ELISA techniques to measure IgM and/or IgG is simpler, cheaper, and requires less training for laboratory staff; thus these techniques can be applied to much larger numbers of samples. Innis defined a case as a primary infection if the IgM/IgG ratio was greater than 1.78 or as a secondary infection if the ratio was below this level [125]. Shu used a similar technique with IgM/IgG ratios but with a different cutoff, 1.2, while Kuno used the cut-off of 1.4 [126, 127]. The problem with these algorithms is that they do not account for the well-established evolution of antibody kinetics during acute infections. In some studies, a combination of the two cutoffs has been used, i.e.

a case was defined as primary if the IgM/IgG ratio was above 1.78, secondary if the IgM/IgG ratio was under 1.2 and unidentifiable if the ratio was in between [158]. This combined algorithm may be more relevant because it, to a certain degree, takes into account the evolving antibody response; however a proportion of cases where the ratio falls between the two cutoffs cannot be classified. Other algorithms have used IgM/IgG ratios with paired samples, IgG titers, and IgG avidity, but all these methods have issues related to inconvenience with sampling and/or complicated methodology [112, 128, 159, 160]. Another disadvantage of algorithms based on IgM/IgG ratios is that typically they rely on in-house assays and these tend to be quite variable, especially between different laboratories. In 2009, Cordeiro developed an algorithm based on a commercial kit (Panbio Capture IgG) with cutoffs depending on the day of illness [129]. However, the number of samples included on each day of illness was small. Also the model used a combination of virology and serology results as the gold standard to discriminate primary from secondary dengue, including the Panbio Capture IgG which was later used as the main parameter in the model.

3.2 Purpose of the study

The aim of this study was to develop models suitable to distinguish primary from secondary dengue with high accuracy using serological data from single specimens obtained on any day of illness during the acute phase.

3.3 Materials and Methods

3.3.1 Recruitment sites

The main site for patient recruitment for the studies described was the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City. This is the referral hospital for infectious diseases for southern Vietnam and serves a catchment population of 38 million people, under the direction of the Ministry of Health of Vietnam. HTD has 21 clinical and ancillary departments with about 500 beds. HTD is the founding partner and host of the Oxford University Clinical Research Unit, where I am doing my PhD programme. Some patients were also recruited from a community clinic operating in District 8 of the city. This clinic is run by an experienced physician who also has a position at HTD and has been involved with clinical studies about dengue for more than 10 years.

3.3.2 Study population

All confirmed dengue patients (aged 5-25 years old) who had been/were enrolled into one of our ongoing dengue studies in Ho Chi Minh City, Vietnam (Table 3-1) within the six months prior to the start of this study in 2010 up to the study closure in 2013, were contacted and asked to join this study and attend a follow-up visit approximately 6 months after the acute illness. All the acute dengue studies had been carried out with appropriate local and international IRB approvals, and the protocol for this study was approved by the Ethical Committee of HTD and the Oxford Tropical Research Ethics Committee. Following written consent by the patient or the patient's parent/guardian a short questionnaire was completed and a 2 ml blood sample was taken if the subject had been well during the intervening time. In the majority of the primary studies from which these subjects were recruited the patients had been enrolled within 72hrs of fever onset, except for one hospital based study (Study 3) when patients were enrolled up to day 5 of illness [36, 44]. All participants had been reviewed daily from study enrolment until afebrile for 2 consecutive days, or discharge in cases of hospitalization, with detailed clinical information recorded and, as a minimum, a 1ml blood sample was obtained at each visit for a complete blood count with the residual plasma saved for research. Additional blood samples were obtained for other tests according to the individual study protocols. The day of illness was counted as 1 from the reported day of fever onset (Day1, Day2, Day3 etc).

3.3.3 Laboratory methods

The laboratory methods used for the daily acute illness specimens are described in Chapter 2 (Materials and Methods), including methods for the two-step realtime RT-PCR, Panbio IgG Indirect ELISA, in-house anti-E protein IgG indirect ELISA and in-house IgM and IgG capture ELISAs. Convalescent samples obtained at around 6 months were sent to the US for PRNTs at the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, NIH; a brief description of the methods used there is also presented in Chapter 2. For this work I used $PRNT_{60}$ titers – i.e. the reciprocal of the dilution at which the number of plaques was reduced by 60%. For convenience, in this chapter I will refer to the serotype causing the recent infection as the "current" serotype when discussing the $PRNT_{60}$ values six months later, while all remaining serotypes will be referred to as "other" serotypes.

3.3.4 Statistical analysis

Continuous variables are presented as median (inter-quartile range, IQR) while categorical variables are presented as frequency (%) throughout. Continuous variables with right-skewed distributions were log2-transformed prior to the analysis. Univariate logistic regression was used to investigate associations between demographic characteristics and clinical parameters on enrolment day, and immune status as defined by the PRNTs.

Table 3-1: The dengue studies from which the patients were recruited

Study	Description of primary study	Number of patients recruited for 6 month follow-up(*) (2010-2013)
1	Community based descriptive study of children with suspected dengue – 2005-2009 Participants: children aged from 5-15 years.	23 (0)
2	Hospital based clinical trial of early steroid therapy for dengue – 2008-2010 Participants: patients aged from 5-20 years	127 (N/A)
3	Hospital based descriptive study of adults with suspected dengue – 2009-2011 Participants: patients aged from 19-25 years	9 (N/A)
4	Community based study focused on diagnosis and risk prediction in early dengue – 2011 onwards	144 (21)

** The numbers represent the total number of participants recruited from each study, and in brackets, for the community-based studies, the number of the subjects who were hospitalized.*

Only cases with clearly defined immune status according to the PRNTs were included in the development of the diagnostic algorithms. First I developed "all-inclusive" models, aiming to incorporate data from all available samples from Day2 – Day7 and to develop a unified algorithm which can determine the immune status based on a single sample from any of these days. The "all-inclusive" models were based on logistic regression with immune status as the outcome, and the (log2 transformed) marker value and the day of illness as predictors. In order to take into account potential dependency between samples from the same patient, marginal logistic regression was used and parameters estimated based on generalized estimating equations and an independence working correlation structure. One model was

developed for each of the following parameters - Panbio Indirect IgG, in-house anti-E indirect IgG, in-house capture IgM, in-house capture IgG, and in-house capture IgM/IgG ratio. Within each all-inclusive model the cutoff point for that parameter was taken as the point where the probability of differentiating primary from secondary dengue achieved the maximum accuracy ([true positive + true negative]/total cases). The coefficients relevant to that marker for each individual day of illness were then used to calculate the day-specific cutoffs with this optimal probability. To be specific, if the logistic model specified a relation of the form logit (probability of detecting secondary infection)=a + b1 * marker + b2 * day of illness, and the optimal cutoff point for the probability was determined as p, then the cutoff for the marker on Day2 = [logit(p) - a - b2 * 2] / b1, the cutoff for the marker on Day3 = [logit(p) – a - b2 * 3] / b1, and so on for each successive day.

Potential interactions between marker levels and day of illness, non-linearity of marker effects (modeled as flexible natural cubic spline functions with 4 degrees of freedom), or effect of other covariates (sex, age, serotype, hospitalization) were assessed using Wald-type tests.

The resulting all-inclusive models were validated using bootstrapping and temporal validation. Bootstrap validation was implemented as described in Harrell, Chapter 5.2.5 [161]. Specifically, I repeatedly fitted the models to 1000 bootstrapped datasets and evaluated them on the original dataset to estimate the "optimism", i.e. the amount of over-fitting obtained from fitting and evaluating the model on the same dataset. Subsequently, the apparent performance of the models was corrected for this optimism. For temporal validation, patients were divided into 5 equal groups by time. I then carried out model development on training sets including 4 of these patient groups on each occasion, and validated the model on the remaining (5th) group of patients. The final performance was calculated as the average performance after 5 times of validation.

The performance of these all-inclusive models was compared to a number of "time-specific" models which included only the corresponding marker values obtained on Day3 (early-phase models); on Day6 (late-phase models); on both Day3 plus Day6 jointly (dual-phase models); and all days from Day3 to Day6 jointly (Day3-6 models) using univariate or multivariable logistic regression. Accuracy, area under the ROC curve (AUC), specificity, sensitivity, positive predictive value and negative predictive value were used in assessing and comparing

the performance of the models. In addition, several of the algorithms currently in use were assessed using this dataset [125, 127].

All statistical analyses were performed with the statistical software R version 3.1.1 (07/10/2014) [162]. Marginal logistic regression models were fitted with the companion R package geepack version 1.2-0 [163].

3.4 Results

3.4.1 PRNT results and discrimination of primary from secondary dengue

A total of 303 patients with confirmed dengue who participated in one of our acute dengue studies, agreed to join this study between May 2010 and July 2013. All the study participants had been well in the intervening time since their acute illness. The median time to this late convalescent visit when the samples for the PRNT assays were obtained, was 212 (IQR: 188-239) days since fever onset.

The $PRNT_{60}$ titer against the current serotype was not always the highest of the values measured at this time. In general the $PRNT_{60}$ titers against DENV1 were higher than the titers against the other serotypes. There were 4 main patterns that I observed: i) pattern 1 – high $PRNT_{60}$ titer against the current serotype with negative $PRNT_{60}$ titers against all other serotypes, using the conventional cutoff of 10; ii) pattern 2 – high $PRNT_{60}$ titers against at least 2 serotypes, including the current serotype; iii) pattern 3 – $PRNT_{60}$ titer below 10 for the current serotype with variable responses to the other serotypes; and iv) pattern 4 – similar and low $PRNT_{60}$ titer (around 20) for the current serotype and one or more of the other serotypes. Although defining immune status for patients with patterns 1 and 2 was straightforward this was more difficult for patterns 3 and 4. In consultation with Dr Whitehead from NIH, I decided on the system presented in Table 3-2, and accepted that not all patients could be classified. First, in all cases a response to the current serotype (as identified by RT_PCR during the acute illness) should be clear, with a $PRNT_{60}$ value $\geq$ 20 documented. If no response was measurable to this serotype the immune status was automatically classified as unknown. Infections were then defined as primary if the $PRNT_{60}$ titers against all other serotypes were <20, or as secondary if the $PRNT_{60}$ titer against at least one other serotype was >40 or >$PRNT_{60}$ titer of the current serotype. All other cases were defined as unknown immune status.

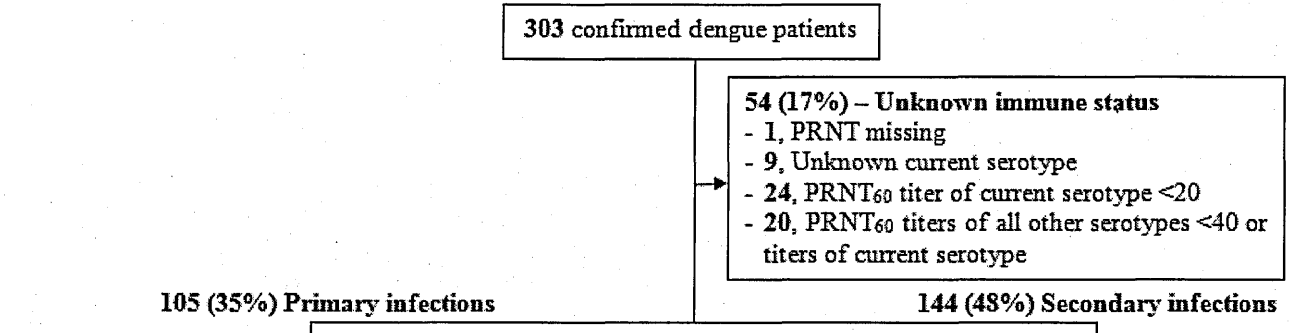
Table 3-2: Rule for defining immune status based on $PRNT_{60}$ results

	$PRNT_{60}$ titers against:	
Immune status	**Current serotype**	**Other serotypes**
Primary	≥ 20	all < 20
Secondary	≥ 20	at least one other ≥ 40 or ≥ the titer for the current serotype
Unknown	< 20	-
	≥ 20	all < 40 and < current serotype's $PRNT_{60}$ titer, but not all <20

Based on these definitions, 105/303 patients (35%) were defined as having primary infections and 144 patients (48%) were defined as having secondary infections. The remaining 54 patients (17%) could not be classified; in 24 patients the $PRNT_{60}$ to the current serotype was below the threshold of 20; in 20 cases with an acceptable response to the current serotype, the $PRNT_{60}$ values for the other serotypes did not fit the definition for a secondary response; 9 patients with serologically confirmed dengue were found not to have had the serotype identified by RT-PCR during the acute illness; and in 1 case the $PRNT_{60}$ results were missing. These data are summarized in **Error! Reference source not found.**.

3.4.2 Patient characteristics and associations with immune status

The majority of the patients enrolled in this project were recruited from 2 of our acute dengue studies, as indicated in Table 3-1. A) Study 2 - the hospital based trial of early prednisolone therapy that ran from 2008 to 2010. Enrolment was limited to subjects with less than 72 hours of fever, and generally occurred within the first 24 hours after hospitalization. No difference was detected in the IgM and IgG levels across the three treatment arms in the trial [164]. B) Study 4 - a community based study focused on diagnosis and risk prediction in early dengue that commenced in 2011 and is still in progress. This study also enrolled within 72 hours of fever onset. Although enrolled as outpatients, participants in this study can be hospitalized if for any reason the treating physician considers this necessary, and a total of 21 patients included in this sub-study were hospitalized.



Current serotype	N	Anti-DENV1 titers	Anti-DENV2 titers	Anti-DENV3 titers	Anti-DENV4 titers
DENV1	75	**251 (135-427)**	-	-	-
DENV2	18	-	**70 (26-122)**	-	-
DENV3	9	-	-	**73 (61-93)**	-
DENV4	3	-	-	-	**44 (33-45)**

Current serotype	N	Anti-DENV1 titers	Anti-DENV2 titers	Anti-DENV3 titers	Anti-DENV4 titers
DENV1	55	**145 (91-300)**	72 (15-165)	30 (14-59)	36 (18-87)
DENV2	38	272 (75-632)	**49 (28-79)**	56 (27-151)	39 (24-62)
DENV3	13	47 (36-215)	18 (<10-113)	**39 (31-138)**	17 (10-138)
DENV4	36	168 (77-505)	34 (18-84)	31 (12-104)	**40 (26-71)**

Figure 3-1: Summary of PRNT results and the classification of immune status

For each immune status category the PRNT titers are summarized in terms of median (inter-quartile range), with the current serotype shown in bold. In the blank cells titers were either immeasurable or low ($\geq 10 < 20$).

Patient characteristics for the subjects with primary, secondary and unknown immune status are presented in Table 3-3. Using univariate logistic regression, older age and female sex were more likely to be associated with secondary dengue, with odds ratios (OR) of 1.11 (95% confidence interval [95% CI]: 1.05 – 1.17) for each +1 year increase in age and 2.79 (1.61 – 4.83) for female gender, respectively. However, there was no significant difference in age between male and female patients. In addition, infections with DENV2 and DENV4 were more likely to be secondary than were DENV1 infections, with OR (95% CI) for DENV2 and DENV4 compared to DENV1 of 3.03 (1.57 - 5.84) and 16.36 (4.79 - 55.88) respectively. Patients with secondary dengue seemed to present later than those with primary infections, though the day of illness at presentation was not significantly associated with immune status (p=0.1, logistic regression). Surprisingly, patients with secondary dengue were less likely to be hospitalized (OR (95% CI): 0.49 (0.29 – 0.82). However this finding likely reflects demographic differences in the different study populations; thus patients in the community studies (mainly Study 4) were more likely to be older than patients in the hospital based studies (mainly Study 2) with a median (IQR) age of 15 (12, 20) years compared to 12 (10, 14) years). Thus a higher proportion of secondary dengue was seen among community participants compared to patients who were hospitalized at any time (67% versus 50%). Considering Study 2 only, being female, older, and having either a DENV2 or DENV4 infection (compared to DENV1) was more likely to be associated with secondary dengue. When assessing Study 4 alone the results were similar, except that later enrolment was also significantly associated with secondary dengue.

IgM and IgG levels from the day of enrolment (Day2 or Day3 in 93% of cases) are summarized in Table 3-4. IgG but not IgM levels were significantly higher in secondary than in primary dengue, after adjustment for the day of illness at enrolment. The kinetics of the antibody responses by day of illness are shown in Figure 3-2, for all three immune status groups. IgG levels started to rise earlier and peaked at a higher level in secondary than primary dengue while IgM kinetics were quite similar between primary and secondary infections. The responses observed in those with

indeterminate immune status were more similar to the secondary than the primary responses.

Table 3-3: Patient characteristics

		Primary N=105 (35%)	**Secondary** N=144 (48%)	**Unknown**(*) N=54 (17%)	p value
Age (years)		13 (10.0-16.0)	14 (11.0-19.0)	14 (12.0-	< 0.001
Sex (male)		79 (75)	76 (53)	42 (78)	< 0.001
Day of fever at enrolment	1	6 (6)	5 (4)	6 (11)	0.1
	2	51 (49)	54 (38)	23 (43)	
	3	45 (43)	82 (57)	25 (46)	
	4	2 (2)	3 (2)	0 (0)	
	5	1 (1)	0 (0)	0 (0)	
Serotype	1	75 (71)	55 (38)	8 (18)	< 0.001
	2	18 (17)	40 (28)	18 (40)	
	3	9 (9)	13 (9)	7 (15)	
	4	3 (3)	36 (25)	12 (27)	

Continuous variables are presented as median (IQR) and categorical variables are presented as number and frequency. P values are from comparisons between primary and secondary infections, using univariate logistic regression.

(*) There were 9 cases with unknown serotypes

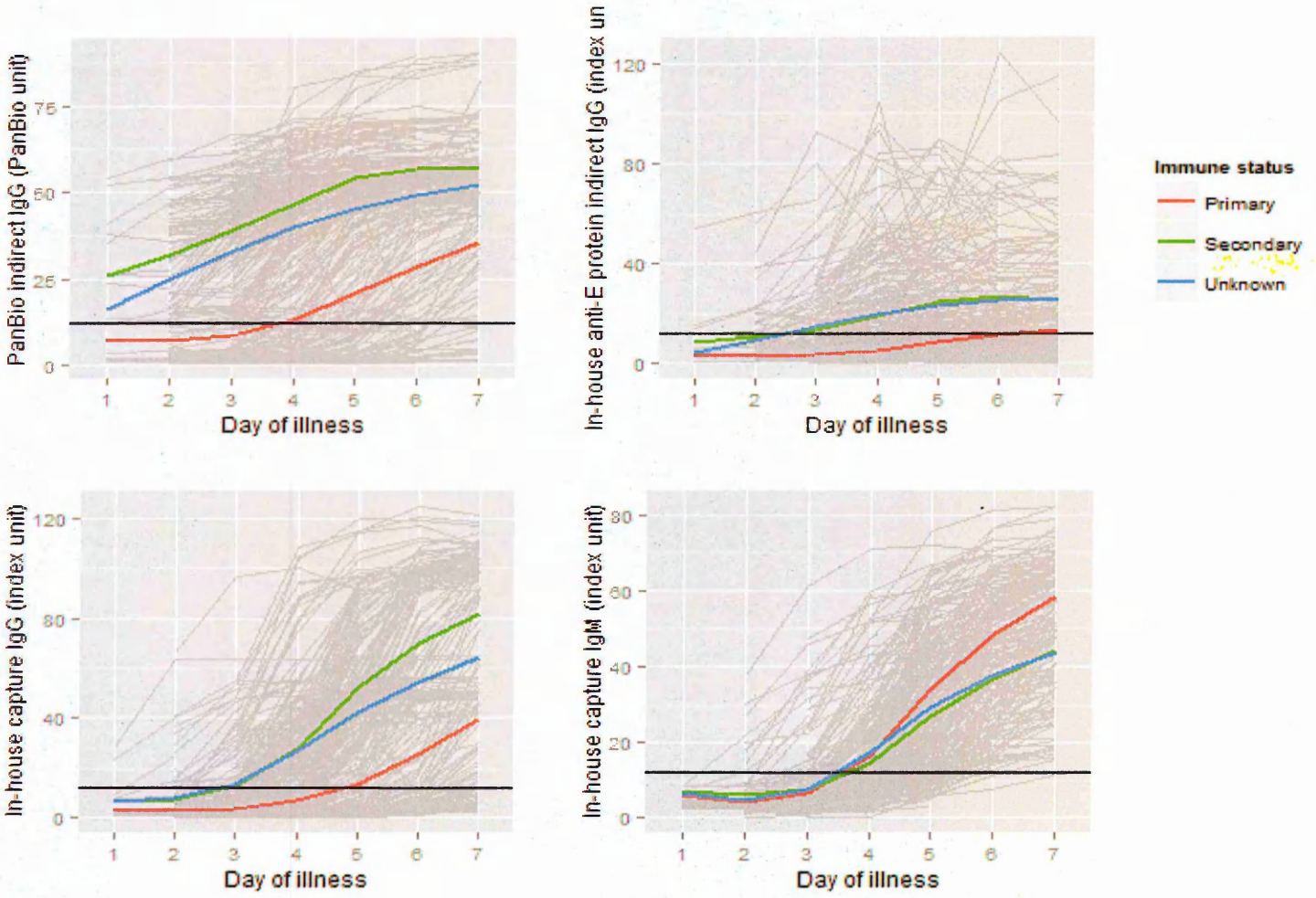


Figure 3-2: Antibody kinetics by immune status. The colored lines represent the smoothed median for each immune status group, while each grey line represents an individual patient's values. The black horizontal lines indicate the cut-off for a positive result for each test.

Table 3-4: IgM and IgG levels at enrolment

Parameter	Primary[1] N=104	Secondary[2] N=135	Unknown[3] N=51	p value
Panbio Indirect IgG (Panbio unit)	2.2 (1.1 - 11.0)	38 (24.8 - 49.5)	33.5 (9.8 - 45.9)	<0.001
In-house anti-E indirect IgG (index unit)	2.1 (1.2 - 3.4)	6.3 (3.1 - 15.5)	5.8 (2.6 - 16.4)	<0.001
In-house capture IgG (index unit)	1.3 (0.5 - 1.9)	6.2 (3.3 - 11.6)	4.7 (1.4 - 11.7)	<0.001
In-house capture IgM (index unit)	4 (2.7 - 5.9)	4.8 (3.7 - 7.0)	4.6 (3.9 - 6.7)	0.5

P values relate to comparisons between primary and secondary dengue, using logistic regression adjusted for day of illness at enrolment. (1) 1, (2) 9, and (3) 3 cases did not have results for these parameters on the enrolment day.

3.4.3 All-inclusive models

Values for the different parameters obtained from Day2 to Day7 (541 and 673 samples from 105 primary and 144 secondary infections, respectively) were used to develop the all-inclusive models. The all-inclusive model based on the Panbio Indirect IgG gave the best performance compared to the other models (Table 3-5). When evaluated separately by illness day, the performance of the all-inclusive model for Panbio Indirect IgG proved to be better in the early phase rather than the late phase, with decreasing accuracy from Day2 to Day7: 0.87, 0.87, 0.87, 0.85, 0.77, and 0.79.

The performance of the all-inclusive models using the in-house capture IgG and in-house capture IgM/IgG ratio were also good, although again with variable performance throughout the illness course (Table 3-5). However, as expected the model based on in-house capture IgM values was not suitable to differentiate primary from secondary dengue with an accuracy of only 0.58.

Table 3-5: Performance of the all-inclusive models to discriminate primary from secondary dengue

Marker	AUC (95% CI)	Accuracy	Sens.	Spec.	PPV	NPV
Panbio Indirect IgG	0.90 (0.88 - 0.92)	0.85	0.91	0.78	0.84	0.86
In-house anti-E indirect IgG	0.80 (0.78 - 0.83)	0.76	0.78	0.74	0.80	0.72
In-house capture IgG	0.86 (0.83 - 0.89)	0.84	0.89	0.77	0.84	0.84
In-house capture IgM	0.55 (0.51 - 0.59)	0.58	0.81	0.28	0.60	0.53
In-house capture IgM/IgG ratio	0.88 (0.85 - 0.90)	0.84	0.90	0.77	0.83	0.85

(*) *The all-inclusive models were developed using marginal regression, clustered by patient, with the following formula: Logit (secondary/primary) = Marker + DOI*

For this and subsequent similar tables in this chapter AUC: area under ROC curve; Sens: sensitivity; Spec.: specificity; PPV: positive predictive value; NPV: negative predictive value

Cutoff was selected to maximize accuracy as described in the statistical methods section.

Using a Wald-type test, an interaction term between marker level and day of illness was shown to have a significant effect in all the all-inclusive models (all p values < 0.001), except for the model based on the in-house capture IgG where the result was borderline (p=0.07). However, the general performance of the various models with or without interaction was comparable, except for the model using the Panbio Indirect IgG. In this instance although the general performance of the two models with and without the interaction was comparable, the performance of the model with the interaction was much better on Day6 (accuracy of 0.77 and 0.84 for non-interaction and interaction models respectively). Adjustment for other covariates (sex, age, serotype, hospitalization and time of sampling), or including the marker in a flexible, potentially non-linear way, both statistically significantly improved on the all-inclusive models (all p values < 0.001 for Wald-type comparison of the more flexible models to the basic all-inclusive models), but the performance of the models changed very little (Table 3-6).

Taking into account these effects, I decided to choose linear models including the marker (modeled as a linear term) and the day of illness (but no further adjustment)

for the in-house anti-E indirect IgG, the in-house capture IgG and the in-house capture IgM/IgG ratio for all subsequent analyses. The same approach was used for the Panbio Indirect IgG but an additional interaction term between the marker and the day of illness was included, which was also used for all subsequent analyses. The performance of the selected all-inclusive models for each individual day from Day2 to Day7 is summarized in Table 3-7.

Table 3-6: Performance of all-inclusive models, adjusted for interactions with day of illness, significant covariates, and non-linearity

Marker	Performance					
	AUC (95% CI)	Accuracy	Sens.	Spec.	PPV	NPV
All-inclusive models with interaction with day of illness						
Panbio Indirect IgG	0.90 (0.88 - 0.92)	0.84	0.89	0.76	0.82	0.85
In-house anti-E indirect IgG	0.82 (0.80 - 0.85)	0.76	0.86	0.64	0.75	0.78
In-house capture IgG	0.88 (0.86 - 0.90)	0.83	0.90	0.75	0.82	0.86
In-house capture IgM/IgG ratio	0.89 (0.87 - 0.91)	0.83	0.86	0.78	0.83	0.82
All-inclusive models adjusted for the covariates (*)						
Panbio Indirect IgG	0.90 (0.89 - 0.92)	0.83	0.90	0.75	0.82	0.86
In-house anti-E indirect IgG	0.83 (0.81 - 0.85)	0.76	0.80	0.71	0.77	0.74
In-house capture IgG	0.89 (0.87 - 0.91)	0.83	0.90	0.74	0.81	0.86
In-house capture IgM/IgG ratio	0.89 (0.88 - 0.91)	0.83	0.85	0.81	0.84	0.81
All-inclusive models adjusted for non-linearity						
Panbio Indirect IgG	0.89 (0.87 - 0.91)	0.84	0.92	0.74	0.81	0.88
In-house anti-E indirect IgG	0.79 (0.76 - 0.82)	0.74	0.76	0.71	0.77	0.71
In-house capture IgG	0.86 (0.84 - 0.88)	0.82	0.90	0.74	0.81	0.85
In-house capture IgM/IgG ratio	0.88 (0.86 - 0.90)	0.83	0.87	0.78	0.83	0.83

(*): the all-inclusive models were adjusted for sex, age, serotype, hospitalization and time of sampling

Table 3-7: Performance of the all-inclusive models on each individual day of illness, from Day2 to Day7

	Performance				
	Accuracy	**Sens.**	**Spec.**	**PPV**	**NPV**
Day2 (56 primary vs 55 secondary dengue)					
Panbio Indirect IgG	0.86	0.91	0.80	0.82	0.90
In-house anti-E indirect IgG	0.71	0.75	0.68	0.70	0.73
In-house capture IgG	0.76	0.87	0.64	0.71	0.84
In-house capture IgM/IgG ratio	0.80	0.78	0.82	0.81	0.79
Day3 (102 primary vs 135 secondary dengue)					
Panbio Indirect IgG	0.86	0.86	0.86	0.89	0.82
In-house anti-E indirect IgG	0.77	0.73	0.82	0.85	0.69
In-house capture IgG	0.83	0.81	0.85	0.88	0.77
In-house capture IgM/IgG ratio	0.80	0.79	0.80	0.84	0.75
Day4 (102 primary vs 136 secondary dengue)					
Panbio Indirect IgG	0.86	0.86	0.90	0.80	0.85
In-house anti-E indirect IgG	0.79	0.79	0.79	0.84	0.74
In-house capture IgG	0.85	0.89	0.80	0.86	0.85
In-house capture IgM/IgG ratio	0.82	0.87	0.77	0.83	0.81
Day5 (101 primary vs 140 secondary dengue)					
Panbio Indirect IgG	0.87	0.94	0.77	0.85	0.90
In-house anti-E indirect IgG	0.78	0.83	0.71	0.80	0.75
In-house capture IgG	0.85	0.94	0.73	0.83	0.89
In-house capture IgM/IgG ratio	0.86	0.89	0.81	0.87	0.84
Day6 (98 primary vs 116 secondary dengue)					
Panbio Indirect IgG	0.84	0.93	0.72	0.80	0.90
In-house anti-E indirect IgG	0.71	0.79	0.61	0.71	0.71
In-house capture IgG	0.81	0.94	0.66	0.77	0.90
In-house capture IgM/IgG ratio	0.85	0.91	0.79	0.83	0.88
Day7 (82 primary vs 91 secondary dengue)					
Panbio Indirect IgG	0.79	0.74	0.84	0.84	0.74
In-house anti-E indirect IgG	0.64	0.65	0.62	0.66	0.61
In-house capture IgG	0.80	0.93	0.66	0.75	0.90
In-house capture IgM/IgG ratio	0.83	0.96	0.68	0.77	0.93

The cutoffs for each parameter on each individual day, as derived from the all-inclusive models, are presented in Table 3-8. For the Panbio Indirect IgG, the in-house anti-E indirect IgG, and the in-house capture IgG, an infection would be defined as secondary if the value equals or exceeds the cutoff for that day, while for the capture IgM/IgG ratio an infection would be defined as secondary if the ratio falls below the cutoff for that day.

Table 3-8: Cutoffs for the selected parameters, derived from the all-inclusive models on each individual day of illness

Day of illness	**Panbio indirect IgG** (Panbio unit)	**In-house anti-E indirect IgG** (Index unit)	**In-house capture IgG** (Index unit)	**In-house capture IgM/IgG ratio**
2	9.3	3.0	1.6	1.8
3	19	4.0	3.1	1.6
4	28	5.3	6.1	1.4
5	37	7.1	12	1.3
6	46	10	24	1.1
7	53	13	47	1.0

To assess consistency in defining immune status in individuals over time I first used data from all the samples from Day2 to Day7, and found that for 81%, 69%, 72%, and 71% of the 303 patients the results remained the same throughout the acute illness for the Panbio Indirect IgG, in-house anti-E indirect IgG, in-house capture IgG, and in-house capture IgM/IgG ratio respectively. As would be expected consistency was better if the analysis was restricted to the time when the individual models demonstrated good performance; thus the daily results agreed in 262/303 (87%) of individuals between Day2 to Day5 for the all-inclusive model based on Panbio Indirect IgG, and in 260/301 (86%) and 258/301 (86%) of individuals for the all-inclusive models based on the in-house capture IgG and in-house capture IgM/IgG ratio respectively when assessed later, between Day4 and Day7. Consistency within the time associated with good performance was also better in cases defined by the PRNT data as secondary (122/144, 85%); (121/143, 85%); (120/143, 84%) than in

cases defined as primary dengue (73/105, 70%); (59/105, 56%); (65/105, 62%) for the all inclusive models based on Panbio Indirect IgG, in-house capture IgG and in-house capture IgM/IgG ratio respectively.

3.4.4 Validation

In order to validate these all-inclusive models, I applied both internal validation using bootstrapping, and temporal validation. For the latter method, there were 50 patients in each group, except one having 49 patients. The performance in terms of the AUC, accuracy, sensitivity, specificity, PPV and NPV of all the models using both validation methods was very similar to the performance of the models during the development process (Table 3-9), indicating that the models were not over-optimized or over-fitted.

Table 3-9: Validation of the all-inclusive models

	Performance				
	Accuracy	**Sens.**	**Spec.**	**PPV**	**NPV**
Bootstrapping validation					
Panbio Indirect IgG	0.90	0.85	0.93	0.75	0.83
In-house anti-E indirect IgG	0.81	0.76	0.83	0.66	0.76
In-house capture IgG	0.86	0.84	0.87	0.80	0.85
In-house capture IgM/IgG ratio	0.88	0.84	0.90	0.77	0.84
Temporal validation					
Panbio Indirect IgG	0.83	0.88	0.77	0.82	0.85
In-house anti-E indirect IgG	0.72	0.78	0.60	0.72	0.72
In-house capture IgG	0.80	0.86	0.75	0.80	0.80
In-house capture IgM/IgG ratio	0.82	0.86	0.78	0.82	0.81

3.4.5 Comparisons with time-specific models

I then compared the performance of the all-inclusive models with the corresponding time-specific models: early-phase, late-phase, dual-phase and Day3-6 models (Table 3-10).

Table 3-10: Performance of early-phase, late-phase, dual-phase and Day3-6 models

	Performance					
	AUC (95% CI)	**Accuracy**	**Sens.**	**Spec.**	**PPV**	**NPV**
Early-phase models (102 primary vs 135 secondary dengue)						
Panbio Indirect IgG	0.89 (0.84 - 0.93)	0.87	0.91	0.82	0.87	0.88
In-house anti-E indirect IgG	0.81 (0.75 - 0.86)	0.77	0.73	0.82	0.84	0.69
In-house capture IgG	0.83 (0.77 - 0.89)	0.83	0.81	0.86	0.89	0.77
In-house capture IgM/IgG ratio	0.82 (0.76 - 0.88)	0.82	0.86	0.76	0.83	0.80
Late-phase models (98 primary vs 116 secondary dengue)						
Panbio Indirect IgG	0.89 (0.85 - 0.93)	0.85	0.93	0.74	0.81	0.90
In-house anti-E indirect IgG	0.73 (0.67 - 0.80)	0.71	0.82	0.59	0.70	0.73
In-house capture IgG	0.85 (0.80 - 0.91)	0.85	0.92	0.77	0.82	0.89
In-house capture IgM/IgG ratio	0.90 (0.86 - 0.94)	0.86	0.94	0.77	0.83	0.91
Dual-phase models (95 primary vs 109 secondary dengue)						
Panbio Indirect IgG	0.90 (0.86 - 0.94)	0.86	0.89	0.83	0.86	0.87
In-house anti-E indirect IgG	0.79 (0.72 - 0.85)	0.74	0.72	0.77	0.78	0.70
In-house capture IgG	0.86 (0.81 - 0.92)	0.85	0.91	0.79	0.83	0.88
In-house capture IgM/IgG ratio	0.90 (0.86 - 0.94)	0.86	0.93	0.79	0.83	0.90
Days 3-6 models (90 primary vs 102 secondary dengue)						
Panbio Indirect IgG	0.92 (0.88 - 0.96)	0.88	0.92	0.82	0.85	0.90
In-house anti-E indirect IgG	0.82 (0.76 - 0.89)	0.79	0.79	0.79	0.81	0.77
In-house capture IgG	0.89 (0.84 - 0.94)	0.88	0.91	0.83	0.86	0.89
In-house capture IgM/IgG ratio	0.93 (0.89 - 0.96)	0.88	0.95	0.79	0.84	0.93

The performance of the all-inclusive models on Day3 (accuracy of 0.86, 0.77, 0.83, and 0.80 for Panbio Indirect IgG, in-house anti-E indirect IgG, in-house capture IgG and in-house capture IgM/IgG ratio respectively) and Day6 (accuracy of 0.84, 0.71, 0.81, and 0.85 respectively) was comparable to the performance of the corresponding early-phase (accuracy of 0.87, 0.77, 0.83, and 0.82) and late-phase models (accuracy of 0.85, 0.71, 0.85, and 0.86) respectively. The performance of the dual-phase and Day3-6 models was a little better (accuracy of 0.86, 0.74, 0.85, and 0.86 for the dual-phase models and 0.88, 0.79, 0.88 and 0.88 for the Day3-6 models respectively).

3.4.6 Comparisons with previous algorithms

According to the manufacturer's instructions, when using the Panbio Indirect IgG ELISA kit, an infection is defined as secondary if the Panbio units exceed 40 and primary if the unit value is below 40, regardless of the day of illness when the sample was obtained. In this study, I found that on Day3, the median Panbio Indirect IgG among the secondary dengue group was 39.9 units, i.e. more than 50% of the secondary infections as defined by PRNT had a Panbio Indirect IgG level below the cut-off at this time. Similarly, on Day4, 25% of secondary dengue cases had a Panbio Indirect IgG level below 40 units. In contrast, 25% and 50% of primary dengue cases had a Panbio Indirect IgG level above 40 on Day6 and Day7 respectively.

Table 3-11: Performance of previously published algorithms on the present dataset

Algorithm	Accuracy	Sens.	Spec.	PPV	NPV
General performance					
Innis, 1989	0.80 (0.78 – 0.82)	0.73	0.84	0.73	0.84
Shu, 2003	0.79 (0.77 – 0.81)	0.80	0.79	0.69	0.87
Combination	0.82 (0.80 – 0.84)	0.78	0.84	0.73	0.87
Performance on Day3					
Innis, 1989	0.76 (0.71 – 0.81)	0.77	0.76	0.63	0.86
Shu, 2003	0.74 (0.68 – 0.79)	0.84	0.68	0.59	0.89
Combination	0.80 (0.75 – 0.85)	0.77	0.83	0.72	0.86
Performance on Day6					
Innis, 1989	0.82 (0.77 – 0.87)	0.69	0.90	0.81	0.83
Shu, 2003	0.84 (0.78 – 0.88)	0.78	0.87	0.79	0.86
Combination	0.82 (0.76 – 0.86)	0.78	0.84	0.75	0.86

As indicated in Table 3-11, with this present dataset, the algorithms of Innis and Shu showed better performance in the late phase (accuracy on Day6 of 0.82 (Innis) and 0.84 (Shu)) compared to the early phase (accuracy on Day 3 of 0.76 (Innis) and 0.74 (Shu)). The combined algorithm, i.e the algorithm using both cut-offs of 1.2 and 1.78, gave stably good performance in both the early and late phases (accuracy of 0.80 and

0.82 on Day3 and Day6 respectively). However 6% of cases could not be classified with this strategy.

3.5 Discussion

Discrimination between primary and secondary dengue is important, especially in pathogenesis research, but it is also relevant for epidemiological surveillance, and it may have a role in clinical practice to identify and target patients at greater risk of developing severe dengue. A number of techniques have been used, with HI and PRNT generally considered to provide the gold standard, but over the last 20 years a number of different options based on simpler tests have been introduced [125-129, 158, 165-170]. Although some of these options have been validated against a gold standard, to date little attention has been paid to the evolving nature of the immune response during infection and the resulting variability in defining immune status based on a fixed rule.

In this study I developed various models to define dengue immune status based on serological responses during the acute illness phase, using the PRNT responses six months later as the gold standard. The dataset is unique in that I was able to follow daily serological responses from over 300 acute dengue infections covering all four serotypes, albeit with low representation for DENV3. The all-inclusive models using the Panbio Indirect IgG, in-house capture IgG, and in-house capture IgM/IgG ratio performed well, both in general over the illness course and when derived on any day from Day2 to Day7. These ELISA based techniques are much quicker and easier to perform than HI or PRNT assays, and based on the results from single acute-phase specimens used with the cutoffs derived from the all-inclusive models I was able to achieve accuracy of 80-85% in discriminating primary from secondary dengue compared to the gold standard. The performance of the all-inclusive models using single specimens was comparable to the corresponding time-specific models. Although the dual-phase and Days3-6 models were a little better, the cost and practical difficulties associated with additional sampling and testing limit the relevance of this approach.

I also assessed the performance of two of the established algorithms using our dataset [125, 127], and showed that while performance was good during the late phase (accuracy above 80% on Day6) there were limitations early in the illness course (accuracy of 0.76 and 0.74 on Day3 for Innis's and Shu's respectively). One explanation may be that although in principle the techniques were similar, i.e. capture IgM and IgG ELISAs, all the assays were in-house, so the reagents and the experimental conditions would have differed slightly between the laboratories. Secondly, most of the samples used to develop the established models were obtained later in the illness course. Finally both Innis in 1989 and Shu in 2003 used HI results from the same time as the test specimens as the gold standard to define immune status – intrinsic interdependence of serological responses obtained at the same time in an evolving immune response is to be expected, and may explain why the accuracy of these algorithms was not so good when assessed against a different gold standard that was independent of the acute phase response. The combined strategy (which has never been formally assessed), using both Innis' and Shu's cutoffs of 1.78 and 1.2, gave better performance in both early and late phases, which was comparable to the performance of the all-inclusive model based on the in-house capture IgM/IgG ratio. This is consistent with the findings for the cutoffs derived from the in-house capture IgM/IgG all-inclusive model, which ranged from 1.8 on Day2 to 1.0 on Day7. However, it must be remembered that using the combined algorithm immune status cannot be defined for patients where the IgM/IgG ratio falls between 1.2 and 1.78.

Another point to consider is that although the all-inclusive models based on the in-house capture IgG and the in-house capture IgM/IgG ratio showed good performance throughout the illness course, actually the IgM and IgG responses as measured by capture ELISA did not rise above the threshold for a positive response until after Day4 in most cases. The reliability of measurements under the positive threshold is questionable. Previous work assessing the performance of the IgM/IgG ratio in the early acute phase of dengue also recognized the problem of negative results (responses below the assay threshold for a positive result) of capture IgM and IgG ELISAs at this stage [126]. In addition, the recommendation from Panbio for interpreting their IgG capture ELISA indicates the need for a high cutoff (22) to

distinguish secondary from primary dengue, with the proviso that this cutoff should be applied to late phase specimens obtained between days 6 and 15 of illness.

It is difficult to see that models based on negative results in a capture ELISA, which assesses acute antibody responses, would be acceptable for defining immune status. In contrast indirect ELISA methodology measures much lower concentrations of dengue specific IgG in plasma, which would be present in people previously exposed to DENV but not in naïve people in the early days of an infection, later rising as the acute antibody response is superimposed on pre-existing antibody levels. In line with this, the all-inclusive model based on the Panbio Indirect IgG without the interaction with day of illness showed very good performance in the early phase, with daily cutoffs always above the negative threshold as defined by the manufacturer, but less good performance later in the illness course. By the time the acute IgG response to the current infection becomes established the range of responses is broad, as seen in Figure 3-2, and consequently the ability to discriminate between primary and secondary infections is impaired. It is also clear that use of the recommended cut-off of 40 for the Panbio Indirect IgG without regard to day of illness at sampling, would lead to incorrect interpretation of immune status in many cases. Although the interaction term helped to improve the performance of this model, in practice a convention where an algorithm based on the Panbio Indirect IgG is recommended for early acute specimens, while an algorithm based on capture IgG or IgM/IgG ratios is used for late acute specimens is likely to be more generally preferred. Unfortunately the performance of the in-house anti-E indirect IgG assay was worse than the Panbio Indirect IgG, possibly because the target, i.e. anti-E protein IgG, is present at lower concentrations than the Panbio target, anti-whole virus IgG. In-house anti-E indirect IgG levels rose above the assay positive threshold later than the corresponding Panbio anti-whole virus IgG levels, and by this time the influence of the superimposed acute response obscured differences between the immune status groups.

Several potential limitations to this study need to be considered. Firstly, I developed a large number of different models using this dataset. Sample size estimation is not feasible for this kind of study but in the main analysis I included almost 250

individual patient sample sets, comprising daily acute specimens for an average of 5 days each, making this the largest assessment of its kind to date. I also planned all the statistical analyses in advance, with the main focus on the all-inclusive models, and included validation using bootstrapping and temporal validation to show that the reported models did not over-fit the data or lead to over-optimistic performance claims. One reason for the large number of models is that I wanted to develop algorithms based on both in-house and commercial assays. In-house tests are routinely used in our laboratory as very large numbers of specimens are processed each year; thus, I wished to develop practical tools based on our in-house capture IgG ELISA and/or the capture IgM/IgG ratio to apply to our extensive datasets. However, given the general limitations of algorithms based on in-house assays, where variability in assay performance is to be expected between laboratories and over time, commercial kits that employ standardized reagents and methodology (but are much more expensive than in-house assays), are preferred when there are no financial constraints. Therefore I included assessment of the commercial Panbio Indirect IgG kit, aiming to develop a model that could be applied more widely for dengue studies in other settings using standardized methodology. Although I would have liked to also assess the Panbio Capture IgG and their commercial IgM/IgG ratio this was not possible within the available budget.

Secondly, I used PRNTs as the gold standard for developing the models, not HI as has been used typically in previous studies. One reason for this choice was to try to avoid circularity – i.e. use of the same samples collected at the same time point both for developing the diagnostic algorithms and for the gold standard (HI) assays. In addition, cross-reactivity is an issue for all serological tests for dengue, and in fact the neutralization assay is considered to be the serological technique with the greatest specificity if performed in late convalescence, which could differentiate flavivirus infections (in review of Maeda [171]). In one specific study the neutralizing antibody level against DENV was not influenced by previous vaccination against YFV and JEV [172]. So, by doing the PRNT assays 6 months after the acute illness I tried to minimize the effect of cross-reactive responses elicited during the acute phase. However, although unlikely, occurrence of an asymptomatic dengue infection during

this time in some cases is possible. Several groups have reported variability in the results of PRNT assays, influenced by a number of factors such as the virus strains used or the passage number, the cell lines, and/or the methods used to fit models [173, 174]. In this study, the PRNT assays were all performed in the NIH laboratory of Dr Whitehead, where this technique is regularly performed and standardized. Studies performed in the same laboratory have shown clear differences in responses between subjects immunized with candidate vaccines active against one or more serotypes [124]. Thus I am confident that the technical aspects of the assays were well executed. The 6-month PRNT data were clear with primary infections, but rather complicated to interpret for secondary infections. Given the established cross-reactivity between different serotypes a broad range of responses is to be expected in a population living in an endemic area that may have had multiple exposures. By setting the cutoffs as I did I was conservative about our "gold standard", although this did result in exclusion of 17% of cases from the main analysis. In the 54 patients that were excluded from analysis, the proportion of DENV2, DENV3 and DENV4 infections was higher than DENV1. One possible explanation may be that the virus strains used in the PRNT assay were dissimilar to the strains that are currently circulating in southern Vietnam. This might lead to a bias in the PRNT results, potentially with higher PRNT titers against DENV1 compared to other serotypes, and as a consequence more DENV2, DENV3 and DENV4 infections would be excluded from the analysis. Although a bias of this nature might affect the performance of the all-inclusive models. applying the all-inclusive models to the acute specimens from this "indeterminate" group of 54 patients resulted in the majority (70% - 74%) being classified as secondary infections, which is in agreement with visual inspection of the plots for the various markers by day shown in Figure 3-2. In addition, using the models to define immune status for all 303 patients who participated in the study showed that 59% – 63% were classified as secondary dengue, which is a very similar proportion to the 144/249 (59%) classified as secondary dengue based only on the 6 month PRNT data, suggesting that the "indeterminate" group were similar to the whole study group. There are no published datasets of similar late assessments in known dengue cases to compare with, except one prospective study in Indonesia showing the PRNT titer before and after

symptomatic dengue in 11 children (10 days - ~1year) [175]. When I applied our rule to their PRNT data, immune status was correctly assigned in all 11 patients [175]. In summary, while I would recommend assessing the all-inclusive models against a new gold standard to define immune status (potentially a new PRNT assay using currently circulating virus strains) I believe that the current data are acceptable for my analyses.

The only other flavivirus known to circulate locally is JEV. Previously the virus was endemic in southern Vietnam but over the last 10 to 15 years the number of symptomatic cases seen at HTD has decreased markedly, likely reflecting societal and environmental changes, and possibly the effects of vaccination as well. Vaccination against JEV is now available in Ho Chi Minh City, but as it is not part of the Expanded Program of Immunization it is not free and there is little information on uptake. Although no study participant had a history of encephalitis it is possible that some participants had experienced asymptomatic or pauci-symptomatic JEV infection in the past, or that some participants may have received JEV vaccine. However as the PRNT panel did not include measurement of anti-JEV antibodies the contribution of cross-reactivity to this virus remains unknown. Algorithms developed on the basis of these data should be applicable for use in environments with similar JEV exposure, but may not be relevant where the JEV epidemiology and/or vaccine exposure is different. This may be particularly important with respect to the indirect IgG assays, and there is evidence from sero-surveillance studies on travelers that vaccination with JEV or YF prior to travel can lead to false positive results on dengue indirect IgG tests [120, 176]. However it has been suggested that use of a different cutoff could improve differentiation in these circumstances [177]. Despite this, in our context the all-inclusive model based on the Panbio Indirect IgG performed well against the gold standard PRNT at 6 months suggesting that cross-reactivity with JEV was not a major issue.

In summary, in this chapter I have presented my work to develop diagnostic algorithms aimed at differentiating primary from secondary dengue during the acute illness phase. The variety of systems currently in use by different groups around the world reflects the general feeling that none of them are entirely satisfactory, and

highlights the need for a more effective algorithm. Of course no diagnostic method can ever be 100% sensitive and specific, particularly when the gold standard has limitations, but it is important to understand the confines within which any test has acceptable accuracy. By describing the acute serological responses in fine detail in this large group of confirmed dengue patients this work has contributed to our overall knowledge of the typical patterns seen, and is helpful in defining the general limitations associated with attempting to define immune status during the acute illness. For our use in Vietnam the daily cutoffs I have derived from the all-inclusive models based on the in-house capture IgG and the capture IgM/IgG ratio should prove useful and cost-effective, particularly after day 4-5 of illness. However given the difficulties of generalizing in-house assays in the wider context, the all-inclusive model based on the commercial Panbio Indirect IgG kit is more suitable for defining immune status for general pathogenesis research, especially in the early phase before day 4-5 of illness. Ideally I would have liked also to assess the accuracy of commercial capture ELISAs in the late acute phase, but financial constraints precluded daily testing of all the specimens with such kits to define day specific cutoffs. Given that the participants in the studies described here were mainly children and young adults, reflecting the epidemiology of symptomatic dengue in southern Vietnam, ideally the generalizability of the Panbio Indirect IgG algorithm should be tested in other populations with different endemicity and a wider age range, and if so it might be possible to pursue a targeted assessment of commercial IgG and IgM assays focused on the late acute phase.

Chapter 4

PLASMA VIREMIA KINETICS IN DENGUE; THE INFLUENCE OF SEROTYPE AND IMMUNE STATUS, AND ASSOCIATIONS WITH CLINICAL OUTCOMES

4.1 Introduction

In addition to immune status, plasma viremia is considered to be one of the crucial factors contributing to dengue pathogenesis and the overall severity of disease. It is also commonly used as an endpoint in clinical trials of dengue therapeutics [36, 130]. However, as yet the magnitude and kinetics of plasma viremia are poorly understood, especially with respect to differences by serotype, or to relationships with immune status. Although there have been several studies examining these relationships in dengue patients in the past, the results have been rather inconsistent, at least in part due to insufficient overall case numbers being enrolled to allow differences between subgroups of patients to be reliably assessed [37 , 38-45]. In addition little attention has been paid to the time point in the evolution of the disease when the measurements were obtained – yet it is clear that plasma viremia differs markedly between and within subjects according to the day of illness. Finally, although there are limited data available for DENV1 and DENV2, in general reporting higher viremia in DENV1 than in DENV2, there is very little information published for the other serotypes.

For a number of years our group has been carrying out studies to characterize the clinical phenotype of dengue, recruiting patients from the early stage of fever and following them daily throughout the evolution of their illness, whether managed as outpatients or hospitalized because of worsening clinical status. As well as clinical data, in most case we have serial daily samples available for examination of viral and immunological characteristics. Therefore I planned to use these samples to obtain a detailed picture of the magnitude and kinetics of plasma viremia during the acute phase for the three main serotypes DENV1, DENV2, and DENV3, circulating in

Vietnam between 2006 and 2008, and to correlate these findings with data on the clinical phenotype of the patients. Unfortunately DENV4 has been rather uncommon in Vietnam in recent years, and has only been identified as a significant contributor to the local disease epidemiology since 2012, so I was not able to include assessment of this serotype in my study.

To properly understand these relationships, an accurate assessment of immune status is necessary, and in our previous experience the methods available have been unreliable. In Chapter 3 I described a number of models that I developed to differentiate primary from secondary dengue, derived from analysis of daily acute serological responses in 249 well-characterized dengue patients for whom immune status was assigned on the basis of PRNT results obtained 6 months after the illness episode. Although the model based on the Panbio Indirect IgG demonstrated the best performance with respect to this independent gold standard, this test was not included in the diagnostic work-up of the patient groups available to me for this study. In-house IgM and IgG capture ELISAs are routinely performed for all participants in our dengue research studies at OUCRU. As reported in Chapter 3 I found that the performance of the in-house capture IgG alone, and the in-house capture IgM/IgG ratio was comparable, with an overall accuracy of 0.84 for both models. However, when each illness day was considered separately the performance of the model based on the capture IgM/IgG ratio was more stable, as the accuracy of the in-house capture IgG model was rather reduced in the early acute phase (accuracy 0.76 on Day2). Therefore I decided to use the all-inclusive model based on the in-house capture IgM/IgG ratio to distinguish primary from secondary dengue for the study of viremia kinetics described here.

4.2 Purpose of the study

The aim of this study was to investigate the kinetics of plasma viremia for the dengue serotypes (DENV1, 2, 3) circulating in southern Vietnam between 2006 and 2008, in regards to immune status and association with clinical parameters, using a stored sample bank from over 950 acute dengue cases followed during that time period.

4.3 Materials and methods

4.3.1 Patients and samples

The data and samples used for this study originated from two prospective descriptive studies of dengue in children, aged 5 – 15 years, which took place in Ho Chi Minh City, Vietnam, between 2006 and 2008: one focused on out-patients while the second study recruited hospitalized patients. These studies have been described elsewhere and were approved by both the Ethical committee of HTD and the Oxford Tropical Research Ethics Committee [44]. Briefly, children with suspected dengue seen at one of three clinic study sites across the city, or admitted to the HTD, within 72h of fever onset were eligible for enrolment providing a parent or guardian gave written informed consent and children over 12 years gave assent. Out-patients were seen daily by study staff until afebrile for two consecutive days while hospitalized patients was monitored daily until discharge. Detailed clinical information was recorded in a standard format and a 1ml EDTA blood sample was obtained for clinical tests (haematocrit estimation and platelet count), RT-PCR and serological diagnosis. Clinic physicians were responsible for all management decisions independently of the study protocol; if hospitalization was considered necessary the children were admitted to HTD and the daily assessments continued. All patients were invited to attend for review 2-4 weeks from illness onset.

Definitions used in this study are as follows: the day of reported fever onset was defined as day of illness 1 (Day1). The platelet nadir was the lowest platelet count measured between days 3-8 of illness. The percentage haemoconcentration was the percentage increase in haematocrit comparing the maximum value recorded between days 3-8 of illness, to a baseline value taken as the lowest value obtained on or before illness day 2 or after day 14, provided the platelet count on the same sample was more than or equal to 200,000 cells/μl. If there was no appropriate baseline value for an individual patient we used the local population mean, matched for age and sex, and taken from a local database of more than 1000 healthy Vietnamese subjects. Shock and other severe complications were defined according to the WHO 2009 guidelines [1].

4.3.2 Laboratory assays

The laboratory methods used for the daily acute illness specimens are described in Chapter 2 (Materials and Methods), including methods for the two-step realtime RT-PCR and the IgM and IgG capture ELISAs.

The two-step realtime RT-PCR has been demonstrated to be equally sensitive for DENV1, 2, and 3, i.e. detection of similar amounts of the standard for each serotype is equivalent [44]. Serial plasma samples from each patient were assayed on the same day and grouped by serotype, and all assays were done blind to the clinical outcome.

4.3.3 Distinguishing primary from secondary dengue viral infections

The all-inclusive model based on the in-house capture IgM/IgG ratio developed in Chapter 3 was used for defining immune status. A case was defined as primary if the in-house capture IgM/IgG ratio was equal to or exceeded the cutoff for that day, and as secondary if the ratio was below the cutoff for that day (Table 4-1). Usually, each patient had two samples for serology tests: one obtained at enrolment (within 72h of fever onset) and one at defervescence (outpatients) or discharge (hospitalized cases). For those cases with only one sample available for defining immune status (second sample missing or day of illness at sampling was out of the range of Day2 - Day7), immune status was defined based on this single sample. In cases where there were two samples available, both within the defined range of Day2-Day7, the later sample was used to define immune status given the slightly better performance of the model in the later phase of the acute illness compared to the early phase.

Table 4-1: Cutoffs based on the in-house capture IgM/IgG ratio for differentiating primary from secondary dengue

	Day of illness					
	2	**3**	**4**	**5**	**6**	**7**
In-house capture IgM/IgG ratio	1.8	1.6	1.4	1.3	1.1	1.0

4.3.4 Statistical analysis

Demographic information (age, sex, day of illness, days of sequential follow-up, hospitalization), laboratory parameters assessed at enrolment (platelet count (PLT), enrolment haematocrit (HCT)), and clinical outcomes (platelet nadir, percentage haemoconcentration, occurrence and severity of bleeding, and shock) are described by serotype and immune status. The continuous variables are summarized as median (IQR) while the categorical variables are summarized as frequency and percentage.

For viremia, the value obtained on day of illness 3 (Day3_viremia), the proportion of participants remaining viremic on day of illness 6 (Day6_viremia), the area under the curve for the serial viremia measurements obtained between days 3-6 of illness (AUC Day3_6), the highest observed value of viremia (max_viremia), the day of illness at the max_viremia, and the time of viral clearance were summarized. The time of viral clearance was defined as the day of illness on which the RT-PCR first became negative and remained negative. In all cases viremia was log10 transformed before doing the analysis. All the comparisons were predefined. Viremia parameters were compared by immune status (primary vs. secondary infections) within serotypes (3 comparisons) and by serotype (DENV1 vs. DENV2 vs. DENV3) within immune status (6 comparisons, 3 for primary, and 3 for secondary infections). To account for multiplicity due to the 9 pair-wise comparisons, p-values and associated confidence 95% confidence intervals were adjusted using a single-sep method to protect the family-wise error rate. Of note, this achieves the same goal as the familiar Bonferroni method (which would imply multiplying the raw p-values by 9) but makes more efficient use of the correlation between the different pair-wise comparisons. These multiplicity-adjusted comparisons were implemented based on regression models which included the group, i.e. the combination of serotype and immune status, as a covariate using the companion R package multcomp v 1.3-7 [178].

To assess associations of viremia with clinical outcome measures I chose the Day3 viremia level, as this was available for almost all participants, and examined relationships with outcomes such as the platelet nadir, overall percentage haemoconcentration, and progression to shock.

Linear regression was used for continuous outcomes, logistic regression was used for categorical outcomes and Cox regression was used for time to viral clearance. In addition, as participants were from 2 different studies, all the analyses were adjusted for study. Day of illness at enrolment was adjusted in analyses of max_viremia and the day of max_viremia. Interactions between immune status and DENV serotype were also investigated for the analyses of associations between viremia level on Day3 with clinical outcomes. All analyses were performed using R, version 3.1.1 [162].

4.4 Results

4.4.1 Patients' characteristics

In total, 631 and 321 confirmed dengue patients with fever <72h were enrolled in the hospital and community based studies respectively, between 2006 and 2008. In view of the low numbers, patients infected with DENV4 were excluded. Similarly, those for whom a serotype could not be identified, or where more than one serotype was found, and patients for whom the immune status could not be defined given the information available, were excluded from the analysis (Figure 4-1). Characteristics of patients from each study are summarized in Table 4-2.

Eight (3%) patients from the community study were hospitalized for monitoring. The proportion of patients with primary and secondary dengue was quite similar in the hospital and community studies (the proportion of secondary cases was 50% and 51% respectively). DENV1 predominated in this analysis as well as in southern Vietnam during this time period. There were more DENV3 infected patients in the community study than in the hospital study (the proportions of DENV1, DENV2, and DENV3 in the hospital study were 67%, 19%, 14% and in the community study were 63%, 14% and 23% respectively). In both the community and the hospital based patient groups, DENV2 infection was more likely to be associated with secondary dengue than the other serotypes. Characteristics of all the patients from both studies are summarized by immune status and serotype in Table 4-3.

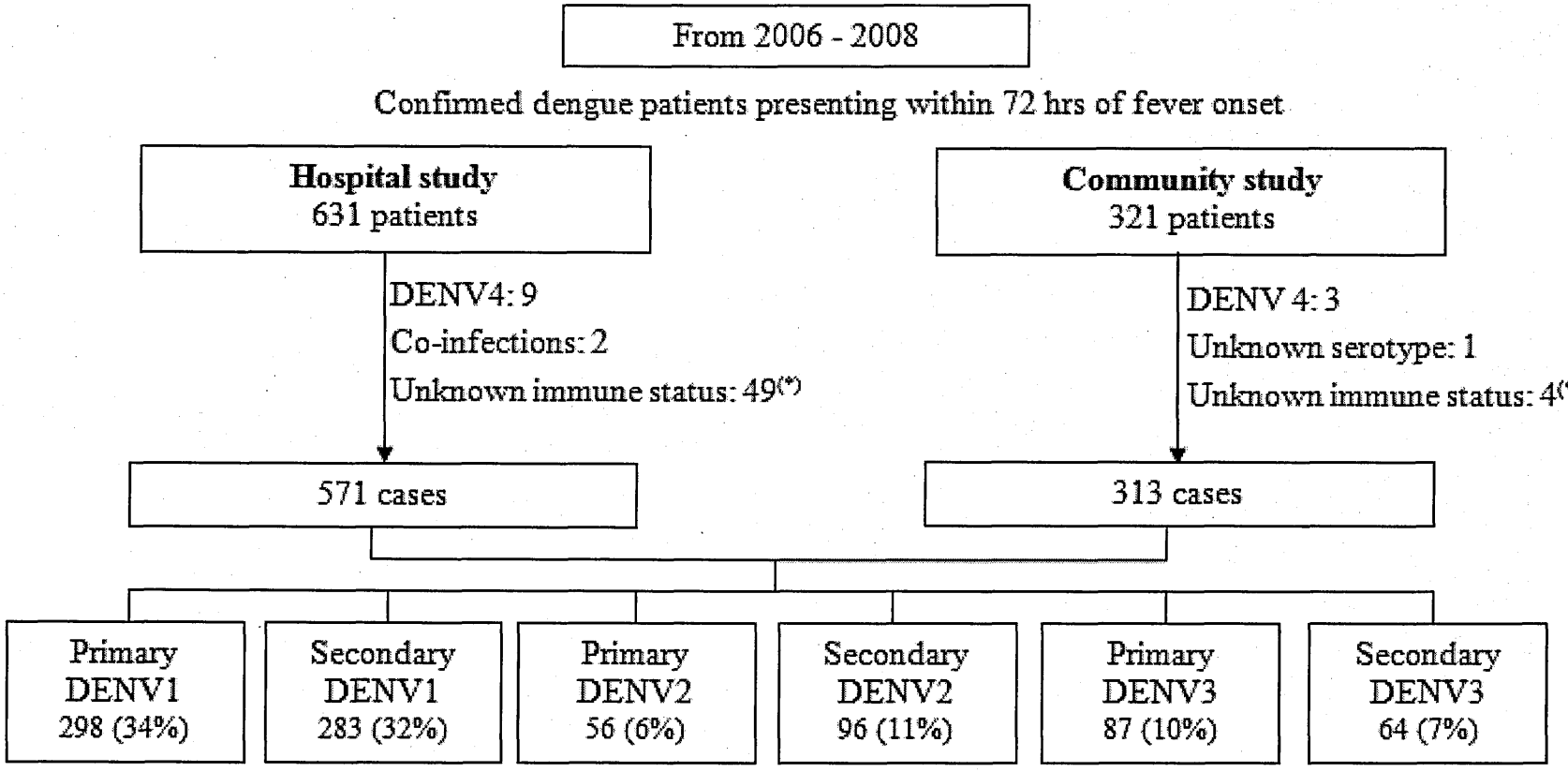


Figure 4-1: Flow chart of patient enrolment

(*) In 42 cases different serological techniques were used during the original study, which did not allow differentiation of primary from secondary infections. In the remaining cases immune status could not be defined because of missing serology results or the fact that the samples had been taken outside the specified range of days.

Table 4-2: Characteristics of patients from the hospital and community studies

Study		**Hospital study**	**Community study**
Number		571	313
Patients hospitalized		NA	8 (3)
Serotypes	DENV1	384 (67)	197 (63)
	DENV2	107 (19)	45 (14)
	DENV3	80 (14)	71 (23)
Immune status	Primary	287 (50)	154 (49)
	Secondary	284 (50)	159 (51)
Demographics			
Age (years)		12 (10 – 13)	11 (9 – 14)
Sex (Male)		383 (67)	165 (53)
Day of illness at enrolment	1	12 (2)	55 (18)
	2	165 (29)	147 (47)
	3	394 (69)	111 (35)
Enrolment parameters			
Platelet (1,000 cells/μl)		162 (121 – 208)[(a)]	187 (151 – 230)[(b)]
Haematocrit (%)		39.0 (37.2 – 41.5)[(a)]	38.8 (36.6 – 41.1)[(b)]
Bleeding at enrolment		113 (20)	13 (4)
Skin bleeding only		94 (17)	5 (1.6)
Mucous membrane bleeding		19 (3)	8 (2.6)
Clinical outcomes			
Days of sequential follow up		5 (4 – 6)	5 (4 – 5)
Platelet nadir (x1,000 cells/μl)		73.6 (45.3 – 110)	98.0 (70.0 – 139)
Day of illness at platelet nadir		6 (5 – 7)	6 (5 – 6)
Percentage haemoconcentration (%)		14.1 (8.0 – 22.5)	11.9 (6.0 – 19.6)
Development of shock		36 (6)	3 (1)
New bleeding after enrolment		263 (46)	206 (66)
Skin bleeding only		231 (40)	190 (61)
Mucous membrane bleeding		32 (6)	16 (5)

The continuous variables were presented as median (IQR) while categorical variables were presented as number (%).
There were a) 3, b) 9 missing values.

There were no major differences in demographic parameters among the groups. There were more male (62%) than female patients (38%) in both studies. The median age of the patients overall was 12 (IQR 9 - 13) years. Although in the hospital study, age did not differ between the primary and secondary dengue cases, in the community study, secondary dengue patients were more likely to be older than primary dengue patients (median (IQR) = 11 (8 – 13) and 12 (10 – 14) years for primary and secondary dengue respectively).

Patients in the hospital study tended to enroll later, with 31% and 65% of patients enrolled to the hospital and community studies respectively before Day3, and were more likely to remain under observation in the study for longer than patients enrolled in the community study ($p < 0.0001$, linear regression, adjusted for day of illness at enrolment). Analyzing the two studies separately showed that a) in the hospital study, patients infected with DENV3 compared to other serotypes presented earlier while b) in the community study, primary dengue cases presented earlier ($p=0.02$ and 0.03 respectively, linear regression, adjusted for immune status or serotype respectively). Within each study (hospital versus community) the numbers of days that patients were monitored did not differ between serotypes or by immune status.

The platelet counts at enrolment were lower in secondary than primary dengue in both studies (median (IQR) = 167,000 (130,000 – 215,000); 156,000 (106,000 – 202,000) for primary and secondary dengue in hospital study; and 190,000 (161,000 – 244,000); 183,000 (140,000 – 121,000) for primary and secondary dengue in community study respectively), but only reached statistical significance in the hospital study ($p=0.003$ and 0.1 respectively, linear regression, adjusted for serotype and day of illness at enrolment). However, the HCT at enrolment was not significantly different between the two immune status groups or between the serotypes (all $p > 0.1$, linear regression, adjusted for serotype or immune status and day of illness at enrolment) in either of the two studies. Considering the whole illness course the platelet nadir was generally lower in the hospital than the community study participants; when split by immune status the platelet nadir was lower in secondary than primary dengue among community participants but not among the hospital study participants ($p<0.001$ and 1.0 respectively, linear regression, adjusted for serotype).

Table 4-3: Patients' demographic and clinical characteristics by serotype and immune status

	Serotype: DENV1		Serotype: DENV2		Serotype: DENV3	
	Primary	**Secondary**	**Primary**	**Secondary**	**Primary**	**Secondary**
Number (% of whole patient group)	298 (34)	283 (32)	56 (6)	96 (11)	87 (10)	64 (7)
Patients enrolled in hospital study	193 (65)	191 (67)	41 (73)	66 (69)	53 (61)	27 (42)
Demographics						
Age (years)	12 (10 – 13)	12 (9 – 13)	12 (10 – 13)	12 (10 – 14)	11 (9 – 13)	11 (9 – 13)
Sex (Male)	184 (62)	180 (64)	35 (62)	61 (64)	52 (60)	36 (56)
Day of illness at enrolment 1	21 (7)	21 (7)	6 (11)	5 (5)	7 (8)	7 (11)
2	110 (37)	84 (30)	16 (29)	35 (36)	40 (46)	27 (42)
3	167 (56)	178 (63)	34 (61)	56 (58)	40 (46)	30 (47)
Enrolment parameters						
Platelet (1,000 cells/μl)	179 [(a)] (145 – 220)	167 [(c)] (119 – 210)	152 [(b)] (123 – 195)	160 [(d)] (106 – 203)	185 (147 – 230)	177 [(a)] (129 – 209)
Haematocrit (%)	39.4 [(a)] (37.0 – 41.8)	39.0 [(c)] (37.1 – 41.5)	38.0 [(b)] (36.9 – 40.2)	39.0 [(d)] (37.3 – 42.0)	38.5 (36.7 – 40.0)	38.9 [(a)] (37.0 – 40.9)

Clinical outcomes						
Hospitalization at any time	195 (65)	193 (68)	41 (73)	67 (70)	54 (62)	29 (45)
Days of sequential follow up	5 (4 – 5)	5 (4 – 5)	4 (4 – 5)	5 (4 – 5)	5 (4 – 5)	4 (4 – 5)
Platelet nadir (x1,000 cells/μl)	84.5 (59.3 – 119)	75.5 (46.4 – 109)	93 (47.7 – 129)	72.5 (43.8 – 131)	104 (70.6 – 136)	84.1 (53.3 – 120)
Day of illness at platelet nadir	6 (5 – 7)	6 (5 – 6)	6 (5 – 7)	5 (4 – 6)	6 (5 – 6)	5 (5 – 6)
Percentage haemoconcentration (%)	14.5 (8.4 – 21.2)	12.9 (7.2 – 21.9)	11.2 (5.3 – 18.2)	14.9 (8.1 – 25.0)	10 (5.4 – 16.3)	10.5 (3.9 – 17.1)
Development of shock	12 (4)	16 (6)	2 (4)	8 (8)	0	1 (2)
New bleeding	167 (56)	149 (53)	25 (45)	49 (51)	45 (52)	34 (53)
Skin bleeding only	145 (49)	137 (49)	24 (43)	42 (44)	39 (45)	34 (53)
Mucous membrane bleeding	22 (7)	12 (4)	1 (2)	7 (7)	6 (7)	0

The continuous variables were presented as median (IQR) while categorical variables were presented as number (%).
There were a) 1, b) 2, c) 3 and d) 5 missing values.

Overall haemoconcentration were not significantly different between the two study groups. However, overall haemoconcentration was generally lower in DENV3 infected patients than in patients infected with other serotypes ($p=0.02$ and 0.04 for hospital and community studies respectively, linear regression, adjusted for immune status).

A total of 3 (1%) and 36 (6%) patients from the community and hospital-based studies progressed to dengue shock syndrome, on average 3 days after study enrolment. No differences were apparent between the serotypes or immune status groups in the development of shock, but the number of events was small. On examination at enrolment, patients from the hospital study had more bleeding, including skin and mucous bleeding than patients from the community study (20% versus 4% respectively). Considering bleeding that developed while under observation, patients enrolled in the community study had more skin bleeding documented than patients in the hospital study (61% versus 40% respectively), but the rate of mucous membrane bleeding (primarily gum bleeding and/or epitasis) were similar. One explanation is that patients from the hospital study presented later than patients from community, so at enrolment, there were more bleedings observed in patients from hospital study. Bleeding at anytime (including enrolment and new bleeding after enrolment) was still a little higher in community study than hospital study (66% versus 58% in community and hospital studies respectively), but mucous bleeding was higher in hospital study (9% versus 5% in hospital and community studies). However no patient in either study experienced severe bleeding requiring any intervention. Differences in the rate of identification of skin petechiae and bruising between the study populations likely reflects the level of attention to detail of the study staff in assessing these features – busy hospital-based study staff may be less likely to document what they see as very minor abnormalities. There was no difference in development of bleeding manifestations between the two immune statuses or among serotypes.

These analyses indicate that the two study populations differed (Table 4-4), with the hospital population generally more severely affected than the community population at the same time point in their illness evolution – likely reflecting the decision (independent of the study) for the subject to be hospitalized.

Table 4-4: Associations between demographic and clinical factors with immune status and DENV serotypes, by study population

Variables	Community study	Hospital study	Whole population
Immune status	The proportion of primary and secondary infection was similar in the hospital and community studies		
Serotype	- DENV1 predominated - There were more DENV3 infections in the community study than in the hospital study - DENV2 were more likely to be associated with secondary dengue than other serotypes.		
Age	Secondary patients (12 (10 – 14) years) was older than primary patients (11 (8 – 13 years)	Age was not significantly different between the two immune status groups or between DENV serotypes	Age was not significantly different between the two immune status groups or between DENV serotypes
Day of illness	Primary dengue cases presented earlier than secondary dengue.	DENV3 infected patients presented earlier than patients infected with other serotypes.	Patients in hospital enrolled later and remained under observation longer than patients in the community.
Platelet count (PLT) at enrolment	Enrolment PLT was not significantly different between the 2 immune status groups or the DENV serotypes.	Platelet count at enrolment was lower in secondary than primary dengue.	Platelet count at enrolment was lower among patients from the hospital study than among those from community study.
HCT	The HCT at enrolment was not significantly different between the two immune status groups or between the serotypes		
Platelet (PLT) nadir	The PLT nadir was lower in secondary than primary dengue among community participants	The PLT nadir was not significantly different between immune status groups or DENV serotypes	The platelet nadir was generally lower in the hospital than the community study participants.
Haemoconcentration	Haemoconcentration was lower in DENV3 infected patients than in patients infected with other serotypes		
Dengue shock	No differences were apparent between the serotypes or immune status groups in the development of shock		
Bleeding	- At enrolment, patients from the hospital study had more bleeding than patients from the community study - While under observation, patients enrolled in the community study had more skin bleeding documented than patients in the hospital study, but the rate of mucous membrane bleeding were similar - No patient in either study experienced severe bleeding requiring any intervention - There was no difference in development of bleeding manifestations between the two immune status groups or among the serotypes		

4.4.2 Viremia kinetics

Enrolment viremia was not significantly different between the two study groups (median (IQR) = 7.4 (6.6 – 8.1) and 7.7 (6.8 – 8.4) log10 copies/ml for the hospital and community studies respectively, p=0.7, linear regression, adjusted for day at enrolment, serotype, immune status). For the main analyses examining relationships with viremia I therefore pooled the data from the two study groups, but, recognizing the differences discussed above in the characteristics of the participants involved, I included an adjustment for the study (hospital versus community) in all comparisons.

A number of parameters describing viremia among the 884 participants involved in the pooled analysis are summarized in Table 4-5, including viremia on day of illness 3 (Day3_viremia), proportion still with measurable viremia on day of illness 6 (Day6_viremia), area under the curve for serial viremia measurements between days 3-6 of illness (AUC Day3_6), the highest observed value of viremia (max_viremia), the day of illness when the max_viremia was documented and time of viral clearance . Viremia kinetics is also presented in Figure 4-2, individually by serotype and immune status.

Comparisons indicate that the viremia kinetics differ significantly according to the immune status for both DENV1 and DENV3, but not for DENV2 infections. Specifically, in DENV1 infection, viremia on Day3, area under the viremia curve from Day3 to Day6, and the maximum observed viremia level, were consistently higher in primary then secondary dengue. Similar results were seen in DENV3 infection, except for the max_viremia, the significance was not reached (p=0.1). The odds of remaining viremic on Day 6 was also approximately 3 – 4 times greater for primary DENV1 and DENV3 infections compared to secondary infections with the same serotype (OR (95% CI = 0.30 (0.18 – 0.53) and 0.27 (0.08 – 0.90) for secondary compared to primary DENV1 and DEND3, respectively). This comparison suggests that viremia generally lasted longer in primary than secondary DENV1 and DENV3 infections (Figure 4-3), which was supported by the analysis for viremia clearance times shown in Table 4-6.

Table 4-5: Summary of viremia kinetics by dengue serotype and immune status

	Serotype: DENV1		Serotype: DENV2		Serotype: DENV3	
	Primary	**Secondary**	**Primary**	**Secondary**	**Primary**	**Secondary**
Number (%)	298 (34)	283 (32)	56 (6)	96 (11)	87 (10)	64 (7)
Day3_viremia (log10-copies/ml)	7.8 [(a)] (6.9 – 8.5)	7.4 [(b)] (6.3 – 8.1)	7.3 (6.3 – 7.8)	7.3 [(b)] (6.4 – 8.0)	7.2 [(a)] (6.4 – 7.8)	6.7 [(b)] (5.4 – 7.3)
AUC Day3_6 (log10-copies/ml)*	16.4 (10.4 – 19.9)	10.7 [(a)] (7.0 – 17.2)	13.0 (8.8 – 17.1)	12.0 (8.0 – 16.1)	14.2 (8.7 – 17.8)	7.8 [(a)] (3.1 – 12.9)
Day6_viremia	119 (44) [(f)]	48 (19) [(e)]	15 (30) [(c)]	20 (23) [(a)]	26 (32)	8 (13)
Max_viremia (log10-copies/ml)	8.0 (7.1 – 8.8)	7.6 (6.6 – 8.3)	7.4 (6.5 – 7.8)	7.6 (6.7 – 8.2)	7.5 (6.8 – 8.0)	7.2 (6.0 – 7.8)
Day of illness at max_viremia (day)	3 (2 – 3)	3 (3 – 3)	3 (2 – 3)	3 (2 – 3)	3 (2 – 3)	3 (2 – 3)
Clearance day of viremia (day)	6 (5 – 7)	5 (5 – 6)	6 (5 – 7)	6 (5 – 6)	6 (5 – 7)	5 (4 – 6)

The continuous variables were presented as median (IQR) while categorical variables were presented as number (%).
There were a) 1, b) 2, c) 3, d) 4, e) 5, f) 9 missing values
Day3_viremia - viremia of day of illness 3, Day6_viremia - proportion of positive viremia on day of illness 6, AUC3_6 - area under curve of the serial measurement during days 3-6 of illness, max_viremia - the highest observed value of viremia.

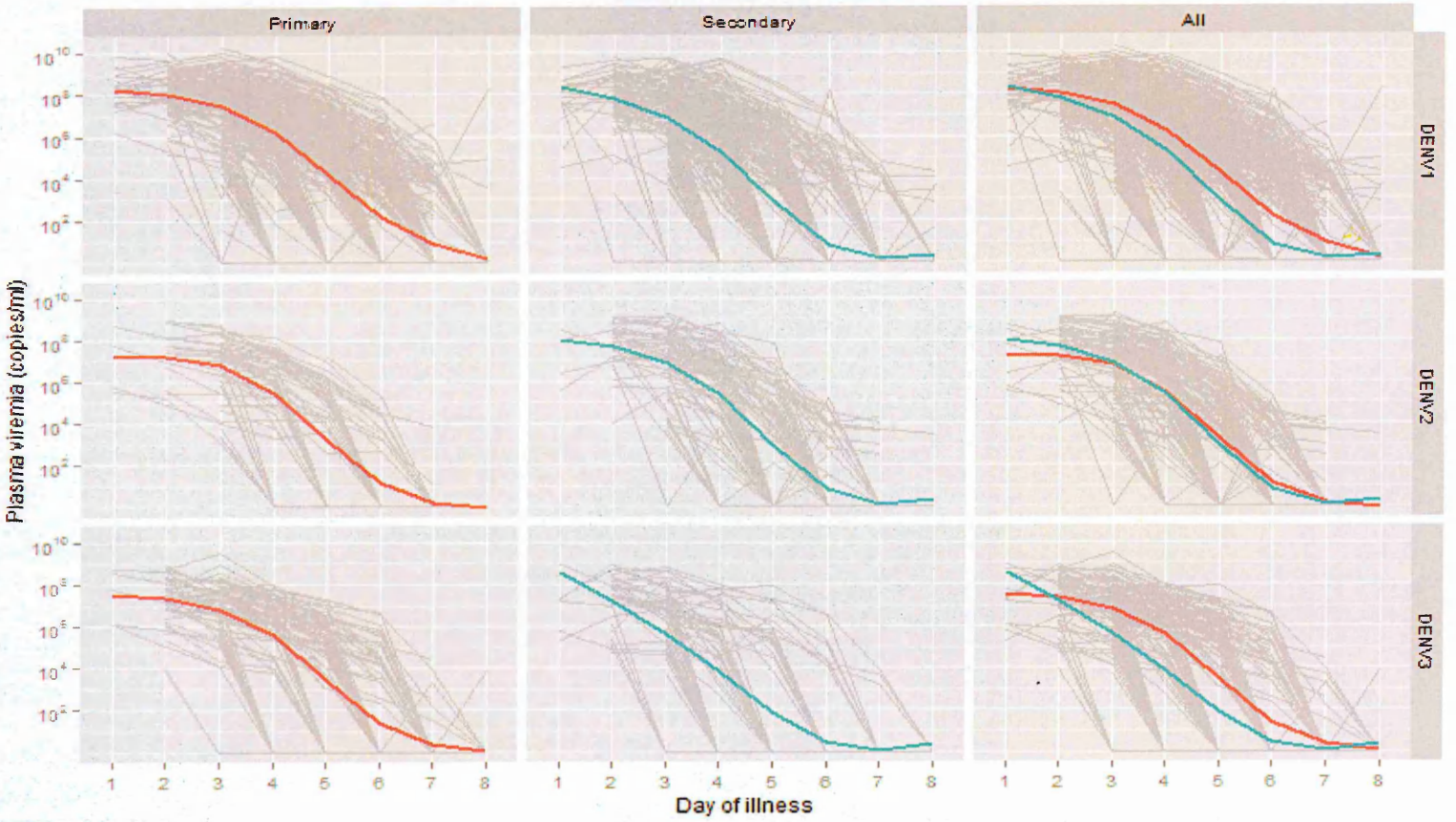


Figure 4-2: Viremia kinetics for 884 study participants by immune status and serotype. The gray lines represent individual patient trajectories while the thick colored lines represents LOESS scatter plot smoothers using local regression.

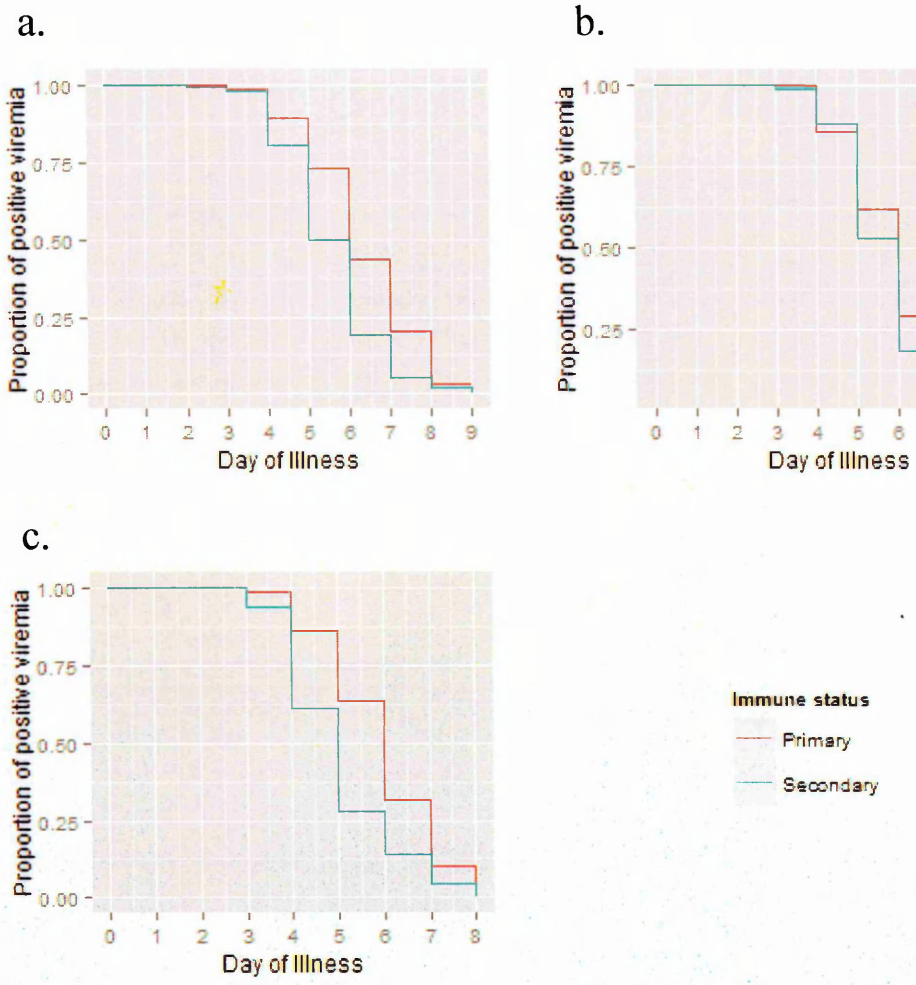


Figure 4-3: Kaplan-Meier plot of plasma viremia. The figure shows a Kaplan-Meier plot indicating the proportion of patients remaining positive for dengue virus by RT_PCR in primary and secondary infections for a) DENV1, b) DENV2 and c) DENV3

The day on which the maximum viremia measurement was recorded did not differ significantly between the two immune status groups in DENV1 and DENV3 infections, but in most cases the maximum viremia was actually recorded on the day of enrolment to the study. There were no significant differences between primary and secondary dengue DENV2 infections for all the parameters assessed (all p values ≥ 0.8).

The results support the view that viremia is influenced by the infecting serotype as well as the immune status of the subject. So I examined the effect of serotype on viremia kinetics by stratifying the analysis by immune status. In primary dengue, viremia was generally higher in DENV1 infections as compared to DENV2 and DENV3, while in secondary dengue the various measures for viremia were generally lowest in the DENV3 infections. Specifically, viremia on Day3, the area under the viremia curve from Day3-6 and the maximum observed viremia were all greater in primary DENV1 than primary DENV2 and DENV3 infections. Primary DENV1 infections also had longer time to viral clearance than primary DENV2 and DENV3 infections, though the statistical significance was not reached. However, I found no difference in the viremia parameters when comparing primary DENV2 and DENV3 infections. With respect to secondary dengue, viremia on Day3, area under the viremia curve from Day3-6, the maximum observed viremia and the time to viral clearance were all higher and/or longer in DENV1 and DENV2 infections than in DENV3 infections. There was no significant difference in viremia kinetics between secondary DENV1 and DENV2 infections (Table 4-7).

4.4.3 Association of viremia on Day3 and clinical outcomes

I used viremia level on Day3 to assess associations with clinical outcomes, as early viremia has the potential to be applied practically in the future if shown to be useful. Higher viremia on Day3 was independently associated both with a lower platelet nadir, and with greater overall haemoconcentration (both p values <0.001, linear regression with platelet nadir or haemoconcentration as outcomes, log10 viremia at Day3, immune status, serotype, interaction of immune status and serotype, study, value (PLT or HCT) at Day 3 as co-variables). With an increase of 1 log DENV RNA copies/ml, the platelet nadir decreased -7,600 (-9,100, -6,100) cell/μl and haemoconcentration increased by 1.25% (0.76 – 1.75). In addition, development of shock was associated with higher viremia on Day3 (p=0.002, logistic regression with shock as the outcome, log10 of viremia at Day3, immune status, serotype, interaction of immune status and serotype, study as co-variables). Thus patients experiencing a dengue infection with the same serotype, immune status and hospitalization status

Table 4-6: Comparison of viremia kinetics between primary and secondary dengue for 884 participants, stratified by serotype

	DENV1		DENV2		DENV3	
	P value	Effect/OR/HR (95% CI)	P value	Effect/OR/HR (95% CI)	P value	Effect/OR/HR (95% CI)
Day3_viremia (log10-copies/ml)	0.02	-0.39 (-0.75; -0.04)	1.0	-0.09 (-0.63; 0.81)	0.02	-0.79 (-1.50; -0.08)
Day6_viremia (yes/no)	<0.001	0.30 (0.18; 0.53)	0.8	0.63 (0.21; 1.86)	0.03	0.27 (0.08; 0.90)
AUC Days3_6 (log10-copies/ml)	<0.001	-3.42 (-4.84; -1.99)	1.0	-0.74 (-3.59; 2.11)	<0.001	-4.83 (-7.61; -2.04)
Max_viremia (log10-copies/ml)	0.006	-0.35 (-0.64; -0.07)	1.0	0.17 (-0.40; 0.74)	0.1	-0.50 (-1.06; 0.06)
Day of illness at max_viremia (days)	1.0	0.00 (-0.12; 0.11)	0.9	0.09 (-0.15; 0.32)	1.0	0.07 (-0.16; 0.30)
Viral clearance	<0.001	1.77 (1.39; 2.27)	0.4	1.09 (0.66; 1.78)	<0.001	2.00 (1.27; 3.17)

Linear regression, logistic regression and Cox regression were used for continuous, categorical and time to event outcomes respectively. Effects/ORs/HRs (hazard ratio) and their 95% CI are presented as appropriate.

In all the comparisons p values and effects/ORs/HRs were adjusted for study and multiple comparisons. Comparisons for maximum observed viremia, and the day on which this occurred, were also adjusted for day of enrolment.

Table 4-7: Comparison of viremia kinetics between serotypes for 884 participants, stratified by immune status

	Primary dengue infections			Secondary dengue infections		
	2 vs 1	**3 vs 1**	**3 vs 2**	**2 vs 1**	**3 vs 1**	**3 vs 2**
Day3_viremia (log10-copies/ml)	-0.64 (-1.26; -0.02)	-0.58 (-1.10; -0.05)	0.06 (-0.67; 0.80)	-0.15 (-0.66; 0.36)	-0.97 (-1.57; -0.37)	-0.82 (-1.52; -0.12)
Day6_viremia (yes/no)	0.60 (0.24; 1.49)	0.59 (0.28; 1.22)	0.98 (0.34; 2.85)	1.23 (0.54; 2.79)	0.52 (0.17; 1.58)	0.42 (0.12; 1.45)
AUC Day3_6 log10-copies/ml)	-2.59 (-5.05; -0.14)	-2.07 (-4.13; 0.00)	0.53 (-2.37; 3.42)	0.08 (-1.95; 2.11)	-3.47 (-5.83; -1.12)	-3.56 (-6.31; -0.80)
Max_viremia (log10-copies/ml)	-0.69 (-1.19; -0.20)	-0.51 (-0.92; -0.09)	0.19 (-0.40; 0.77)	-0.17 (-0.57; 0.23)	-0.65 (-1.13; -0.18)	-0.48 (-1.04; 0.07)
Day at illness at max_viremia (days)	-0.10 (-0.30; 0.10)	-0.13 (-0.30; 0.04)	-0.03 (-0.27; 0.21)	-0.01 (-0.18; 0.15)	-0.06 (-0.25; 0.13)	-0.05 (-0.27; 0.18)
Viremia clearance	1.43 (0.93; 2.21)	1.40 (0.98; 2.00)	0.98 (0.59; 1.61)	0.88 (0.62; 1.23)	1.58 (1.08; 2.32)	1.81 (1.14; 2.85)

2 vs 1; 3 vs 1 and 3 vs 2 mean comparison between DENV2 vs DENV1; DENV3 vs DENV1 and DENV3 vs DENV2 respectively.
Linear regression, logistic regression and Cox regression were used for continuous, categorical and time to event outcomes respectively. Therefore Effects/ORs/HRs and their 95% CI were presented respectively.
In all the comparisons p values and effects/ORs/HRs were adjusted for study and multiple comparisons. Comparisons of max_viremia and the day on which this occurred were adjusted for day of enrolment also.

who had higher viremia had a greater risk of progressing to shock; with an increase of 1 log DENV RNA copies/ml the odd of shock increased 1.63 (1.21 – 2.28) times. Higher viremia on Day3 was also associated with new bleeding which developed after enrolment in general, skin bleeding only and mucous bleeding (all $p \leq 0.03$, logistic regression, adjusted for immune status, serotype and study). Specifically, with an increase of 1 log DENV RNA copies/ml the odds for the appearance of bleeding (both skin and mucous) increased 1.2 (1.1 – 1.3) times; the odds of skin bleeding increased 1.1 (1.0 – 1.2) times; and the odds of mucous bleeding increased 1.3 (1.0 – 1.7) time.

4.5 Discussion

In this study, I confirmed and expanded the current knowledge base regarding the influence of immune status and serotype on the magnitude and kinetics of DENV viremia, by carrying out detailed assessments on almost 900 patients infected with DENV1, DENV2, and DENV3. I also confirmed associations between the magnitude of the viremia response and a number of clinical outcomes (eg platelet nadir and percentage haemococentration), and was able to show for the first time an association between higher viremia during the early febrile phase and subsequent development of DSS. Higher viremia in the early febrile phase was also associated with a slightly greater risk of developing bleeding, both from the skin and mucous membranes.

Previous studies have shown inconsistent results when assessing associations between viremia and immune status, or viremia and dengue severity [37-43]. In addition to generally small sample sizes, viremia was rarely related to the day of illness at sampling in these studies. However, in a previous report from our group involving around 170 patients mainly infected with DENV1 and DENV2, and in a paper examining secondary DENV3 infections in 54 cases, in both of which timing was carefully considered, associations between higher viremia in the febrile phase and a lower platelet nadir and/or greater percentage haemoconcentration during the subsequent course of the illness, were demonstrated [38, 44]. Another factor that has made interpretation of these types of data difficult in the past, has been the method used to classify dengue severity – several groups have used the WHO 1997 classification,

where distinction between DF and DHF is not always clear. This study has the advantage of a much larger sample size than all previous studies, allowing subgroup analysis by serotype and immune status to be performed, as well as rigorous clinical sampling protocols with daily samples obtained from almost all patients during the acute illness. Finally the decision to assess the clinical outcomes separately, with strict definitions used for each parameter, reduces the likelihood of blurred demarcation lines between severity groups.

It is interesting to see that similar patterns, albeit with differing magnitude of responses, were observed for DENV1 and DENV3, but that the pattern of viremia kinetics for DENV2 was rather different. Although DENV1 infections resulted in higher viremia than DENV3 infections generally, the influence of immune status was consistent, with primary infections of the appropriate serotype typically resulting in higher viremia than secondary infections, during the time window when measurements were possible. However the responses observed during DENV2 infections were different; although primary DENV1 infections still demonstrated higher viremia than primary DENV2 infections, this difference was no longer apparent in secondary infections since the viremia level in secondary DENV2 infections remained at a level equivalent to that seen in primary DENV2 infections – i.e. viremia was NOT lower in secondary DENV2 infections. Similarly, although viremia levels were similar between DENV2 and DENV3 primary infections, in secondary infections the DENV2 viremia level was unchanged while the DENV3 viremia was lower, thus making secondary DENV2 viremia higher than secondary DENV3 viremia. Regrettably, so few DENV4 infections occurred during the study period that I was unable to assess relationships to viremia, immune status, or clinical outcomes, for this serotype.

Although these effects of immune status and serotype on viremia kinetics are clear for DENV1, DENV2 and DENV3, the results should be interpreted with caution. In this study, we tried to enroll patients as early as possible in the febrile phase of dengue, and the majority of the patients were actually enrolled on Day 2 or Day 3 of illness. However, only a small number of patients were enrolled on Day 1 of illness (reflecting the health seeking behavior of the local community), at which point high viremia were

already detectable in many cases (Figure 4-2). It is only possible to draw conclusions from the first viremia time-point forward, when in fact the viremia was already declining in most cases. What may have happened in the pre-symptomatic or early symptomatic phase of the illness remains a matter of conjecture; thus viremia may peak before fever onset, or the time from being infected by a mosquito to developing peak viremia may differ by serotype or according to immune status. Unfortunately it is rarely practical to enroll dengue patients earlier in their disease course, in particular in the pre-symptomatic phase, in sufficient numbers to address these questions adequately. One potential area of interest to examine these questions in more detail is human challenge studies. Currently, because of ethical concerns about the potential to increase the risk for severe disease, human challenge studies are conducted in very limited circumstances, generally relating to vaccine development research and usually involving attenuated viruses in naive populations. In the past however, challenge studies using naturally occurring viruses were performed, most notably in the Philippines in the 1920s and by Albert Sabin's group in the 1940s and 50s. In a recent review of Sabin's human challenge experiments, evidence was presented that the incubation period for secondary dengue was shorter than for primary dengue (median incubation time of 4.2 and 6.5 days for secondary and primary infections respectively) [179]. In these experiments, it was not possible to measure viremia but one could hypothesize that in the very early phase of a secondary dengue infection, viremia levels are enhanced by ADE resulting in a rapid rise to peak viremia, potentially earlier than in a primary infection; at the same time the rising viremia triggers an earlier immune response, so that by the time patients present to clinical care the combination of a high early peak viremia in the presymptomatic phase, together with an earlier more aggressive immune response, result in apparently lower plasma viremia levels in secondary cases. The situation with DENV2 appears to be different, but at present it is difficult to propose a theory to explain why this should be.

Another limitation to consider is that the viremia measurements in this study were done on plasma using realtime RT-PCR. Dengue virus has been found in other tissues, and the true contribution of different viral reservoirs (eg. splenic lymphoid tissue, haemopoetic cells) to dengue pathogenesis remains uncertain [12, 14-16, 180]. In

addition, realtime RT-PCR measures total plasma viremia but cannot differentiate between infectious and non-infectious viral particles. Although there is a reasonable correlation between the amount of RNA measured by PCR and quantization of infectious virus by plaque assay techniques, how this correlation is affected by immune status or serotype is unknown [131, 181]. In one study measuring NS1 levels, evidence was presented that NS1 in immune complexes could evade detection in ELISA based assays [182]. Anti–dengue IgG levels were significantly higher in the secondary dengue cases, potentially explaining the lower viremia levels detected in secondary than primary DENV1 and DENV3 infections. However, in the RNA extraction step before performing the PCR, the plasma is processed with strong lytic reagents and at high temperatures, both processes that are likely to separate immune complexes, so I think that the contribution of immune complex formation to the findings observed in this work is likely to be small.

Another limitation to consider is that fact that the participants were from two different studies, one hospital based and one community based. Although the study objectives, study procedures and execution were very similar, inevitably the enrolled patient populations differed slightly, with the hospital population generally being more severely affected than their community counterparts. However, we recognized this difference and were careful to include an adjustment for the study type in all relevant analyses. Another point to stress is that relatively few true severe dengue cases were included, even though the overall study population numbered almost 900 confirmed dengue cases. Estimates vary of the proportion of dengue infections that progress to severe disease, but in our context the figure is thought to be around 3-5% of symptomatic cases who go on to develop DSS. However, in practice many patients do not present to facilities with the capacity to enroll into research studies until late in the disease evolution; we assume that by following a large group of symptomatic patients from the early febrile phase we will capture a representative sample of the clinical spectrum of patients who do subsequently progress to severe dengue, but without a formal cohort design where all subjects from a given area or population are followed actively over a period of some years it is not possible to be certain that the

characteristics of those who develop severe disease are not in some way different to those whom we capture at an early stage of their illness using our current study design.

However, despite these caveats this represents the largest study of its kind to date. In conclusion, I found that the magnitude and kinetics of dengue plasma viremia was influenced by both the immune status of the patient and by the serotype of the current infection. Although what happened before fever onset remains unknown, it is also also clear that viremia in the early febrile phase (Day 3) was associated with several important markers of more severe dengue disease: a lower platelet nadir, higher overall haemoconcentration, and a greater risk for development of shock and bleeding. As quantitative PCR becomes more widely used in clinical practice in the management of other infectious diseases (such as HCV and HBV) the utility of measuring dengue plasma viremia as a potential progostic marker might be considered. Further studies which include viremia as a predictor will need to be conducted to examine this hypothesis in detail, but in endemic areas where healthcare facilities are currently overburdened by large numbers of patients hospitalized for monitoring, then additional prognostic markers, if shown to be robust, would be welcomed.

Chapter 5

EFFECT OF EARLY PREDNISOLONE THERAPY ON DENGUE VIREMIA, NON-STRUCTURAL PROTEIN 1 (NS1) KINETICS AND A RANGE OF IMMUNOLOGICAL CORRELATES IN DENGUE

5.1 Introduction

A small number of anti-viral agents have been trialed to examine their effects for dengue treatment [130, 135, 136]. Although these agents had no significant adverse effects in the trials, they were also not successful in reducing plasma viremia or preventing the development of complications. An alternative strategy aimed at improving outcome is to use immune modulation, such as corticosteroid therapy. Corticosteroids have been used widely to treat diseases where the host immune response is thought to make a significant contribution to pathogenesis. Evidence of benefit has been shown in infectious diseases such as TB meningitis, bacterial meningitis, leprosy and recently, community acquired pneumonia [183-186]. Corticosteroids have been shown to inhibit transcription of pro-inflammatory genes and induce transcription of anti-inflammatory genes [187], and have been demonstrated to suppress both T cell and cytokine responses, which are both hypothesized to contribute to the endothelial dysfunction/vasculopathy that can occur in dengue [188, 189].

In the previous chapter, I described how viremia kinetics differed by serotype and immune status. In this chapter, I will present my work to investigate viremia kinetics in dengue under the effect of an immune modulator belonging to the corticosteroid family, in this case prednisolone. Recently a randomized controlled trial of early oral prednisolone therapy was performed in 225 confirmed dengue cases in Vietnam. Although the trial was primarily designed to assess safety, we did not detect any reduction in the severity of plasma leakage or other recognized complications of dengue [36]. My work looked at the question of whether, by suppressing the immune response in dengue, prednisolone therapy might prolong the plasma viremia and

antigenemia of NS1. In addition, I looked at a variety of factors involved in the immune response, to investigate the effect of prednisolone thereapy at both protein and transcriptional levels in order to try to better understand dengue immunopathogenesis.

5.2 Materials and Methods

Laboratory methods including IgG and IgM capture ELISA, one-step multiplex realtime RT-PCR, qualitative NS1 assays, T cell phenotyping, cytokine quantification, microarray and RT-PCR for validation were performed as described in Chapter 2 (Materials and Methods).

5.2.1 Study population

A randomized placebo-controlled trial assessing the safety of early oral corticosteroid therapy in dengue patients was conducted at the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam, between August 2009 and January 2011 [36]. Ethical approval was obtained from the Ethical Committee of the Ministry of Health of Vietnam and the Oxford Tropical Research Ethics Committee. The trial was registered with the ISRCTN Register (ISRCTN39575233). Once written consent was obtained, 225 patients aged from 6-20 years old with fever for less than 72h and a positive NS1 rapid test (Dengue Duo Rapid Test, SD, Korea) were randomly allocated to oral treatment with either high-dose prednisolone (2mg/kg), low-dose prednisolone (0.5mg/kg), or identical placebo for 3 days (75 patients in each arm). The research blood specimens that are the basis of the results described in this chapter were collected as part of the trial protocol at pre-specified time-points: daily samples from enrolment to discharge were used for viremia quantification and NS1 ELISA assays; samples at enrolment (pre-treatment), 2 days post-treatment initiation, and at follow up (a median of 29 (IQR 27 - 30) days after enrolment) were collected for analysis of plasma cytokines and gene expression microarray; samples at enrolment and discharge (4-11 days post-treatment) were assayed with in-house IgM and IgG capture ELISAs. In addition, samples at discharge were used for analysis of T-cell surface phenotype, and samples 6 months after illness onset were used for $PRNT_{60}$ determinations.

5.2.2 Defining immune status

Because this work was completed before developing the algorithms to differentiate primary and secondary dengue infections that I describe in Chapter 3, I used a system previously developed at our Unit to define immune status, as described elsewhere [36]. In brief, a patient with negative anti-E protein IgG indirect ELISA on the enrolment sample (within 72h of fever onset) and no rise in dengue-reactive IgG (in-house capture IgG) by day 7 of illness was defined as having a primary infection; in contrast, a patient with a positive or equivocal result for the anti-E protein IgG indirect ELISA on the enrolment sample and a rise in dengue-reactive IgG greater than that of the IgM by day 7 of illness, was defined as having a secondary dengue infection.

5.2.3 Microarray data analysis

The raw expression intensity data was extracted from the arrays using GenomeStudio software (Illumina). The raw data was normalized to the background by subtracting the background signal. This software was also used for data quality control (QC). After removing noise by using GenomeStudio, the data was transferred to GeneSpring software (Agilent Technologies) for another standard normalization procedure for one color array data. In brief, the measurements with intensity values of less than 5.0 were corrected to 5.0. Per chip all the signals were normalized to the 80^{th} percentile by dividing each value by the 80^{th} percentile value; per-gene normalization accounted for variability between probe sets for different genes. Only genes that were confidently detected in at least 60% of samples in one of the three treatment groups were used.

The ANOVA t-test developed in GeneSpring Software was used for all comparisons of microarray data. Multiple test correction was done using the Benjamini-Hochberg method. A fold change of 1.5 was defined as the cut-off for screening significant entities. Ingenuity Pathway Analysis software (Ingenuity System, USA) was used to explore the networks, the gene ontology, and the canonical molecular pathways related to the genes identified. A heatmap of hierarchical clustering was drawn with Multi Experiment Viewer software (TM4 Microarray Website Suite). The GEO accession number for the microarray data is GSE40165.

5.2.4 Statistical analysis

The primary comparison between the 3 groups was a linear trend test: a single treatment covariate was included with placebo coded as 0, low-dose as 1, and high-dose as 2. This was based on linear regression for continuous endpoints, logistic regression for binary endpoints and Cox regression for time-to-event endpoints. Comparisons for continuous laboratory parameters were adjusted for day of illness at enrolment and the enrolment value of the parameter.

For virological factors the following parameters were compared: the area under the curve (AUC) for log-transformed serial measurements of plasma viremia obtained between Day3-6 of illness; the number of days from enrolment until the RT-PCR first became negative; and the number of days from enrolment to negative NS1 status. In addition to day of illness at enrolment and the enrolment values, comparisons of viremia and NS1 were also adjusted for serotype and immune status.

For the microarray data I used multivariable linear regression modeling for all comparisons of validated PCR results, expressed as delta Ct values, across and between the treatment arms. In view of the likely evolution of gene expression during the illness episode and the known associations of many of the genes of interest to immune parameters, I adjusted for the absolute neutrophil and lymphocyte counts as well as day of illness at enrolment and the enrolment value. P values for testing of multiple PCR results were corrected using the Benjamini-Hochberg method. The relative expression ratio (R) of the genes between the treatment arms was estimated using the delta delta Ct formula: $R = 2^{-\Delta\Delta Ct}$, with the 95% confidence intervals of R estimated based on the basic bootstrap interval method. Genes with significantly different relative expression ratios across or between treatment arms (i.e. adjusted $p<0.05$ and 95%CI not including 1) were considered up or down regulated as appropriate.

Log transformed values were used for comparisons of cytokine concentrations. IgM and IgG levels and T cell phenotypes at discharge, as well as $PRNT_{60}$ values 6 months after illness onset, were compared between the treatment groups using the Mann-Whitney test. All the analyses were corrected for multiple testing using the Benjamini-Hochberg method.

All analyses other than those pertaining to the microarray data were performed using R - version R2.13.2 [162].

Table 5-1: Baseline characteristics according to treatment allocation

	Placebo (N=75)	**Low-dose prednisolone (N=75)**	**High-dose prednisolone (N=75)**	**All patients (N=225)**
Age [years]	13 (12 - 15)	12 (11 - 14)	12 (10 - 14)	13 (11 - 14)
Gender - Male	56 (75)	54 (72)	51 (68)	161 (72)
Temperature [°C]	38.8 (38.5 - 39.4)	39.0 (38.6 - 39.6)	38.9 (38.5 - 39.4)	39.0 (38.5 - 39.5)
Day of illness at enrolment:				
Day 1	0 (0)	1 (1)	1 (1)	2 (1)
Day 2	28 (37)	23(31)	29 (39)	80 (34)
Day 3	47 (63)	51(68)	45 (60)	143 (64)
Serotype:				
DENV1	41 (55)	46 (61)	49 (65)	136 (60)
DENV2	29 (39)	17 (23)	11 (15)	57 (25)
DENV3	4 (5)	9 (12)	10 (13)	23 (10)
DENV4	1 (1)	2 (3)	4 (5)	7 (3)
Serotype unknown	0 (0)	1 (1)	1 (1)	2 (1)
Immune status:				
Primary	22 (29)	21 (28)	25 (33)	68 (30)
Secondary	43 (57)	39 (52)	35 (47)	117 (52)
Unknown	10 (13)	15 (20)	15 (20)	40 (18)
Plasma viremia [log10copies/mL]	N=75 8.81 (7.98 - 9.23)	N=74 8.77 (8.23 - 9.47)	N=74 8.96 (8.08 - 9.57)	N=223 8.81 (8.05 - 9.39)
Haematocrit [%]	40 (39 - 43)	39 (37 - 41)	39 (37 - 42)	39 (38 – 42)
Platelet count [10^9/L]	143 (108 - 172)	140 (100 - 186)	143 (113 - 201)	142 (107 - 188)
WBC [10^6 dL]	3.8 (2.7 - 5.0)	3.7 (3.0 - 5.4)	4.2 (3.0 - 5.2)	3.7 (2.9 - 5.2)
Neutrophils [%]	67 (59 - 75)	68 (58 - 75)	70 (59 - 79)	69 (59 - 76)
Lymphocytes [%]	18 (13 - 26)	19 (13 - 26)	17 (11 - 29)	18 (12 - 27)

All data are presented as number (%) for categorical variables and median (IQR) for continuous variables.

5.3 Results

5.3.1 Patient characteristics

Between August 2009 and January 2011, 225 participants were enrolled in the study and randomized to one of the three treatment arms. Baseline characteristics were similar in the three treatment groups, Table 5-1. In addition, clinical complications such as dengue shock syndrome (DSS), thrombocytopenia, severe bleeding and coagulopathy occurred at similar rates in the different treatment arms, and at a rate consistent with what is expected in our population. There was no difference in adverse events across the 3 groups, except that a higher incidence of hyperglycemia was noted with high-dose prednisolone therapy (p=0.07) [36].

5.3.2 Viremia kinetics

There was no difference in viremia kinetics between the three treatment groups (Figure 5-1), including both the AUC of log viremia from Day3-6, and the time of viremia clearance (p=0.8 and 0.9 respectively, multiple linear regression adjusted for day of illness at enrolment, pre-treatment value of viremia, immune status and serotype) (Table 5-2).

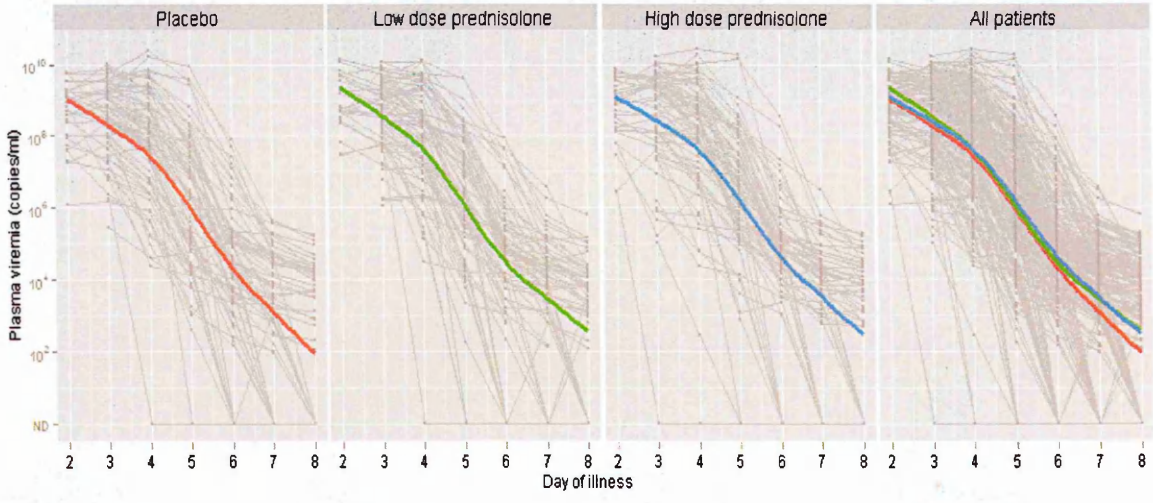


Figure 5-1: Dengue viremia kinetics for all serotypes by day of illness. The figure shows dengue viremia kinetics for all serotypes by day of illness in the three treatment arms separately and finally with all data combined. The gray lines represent individual patient data while the colored lines correspond to loess scatter plot smoothers.

Table 5-2: Effect of early prednisolone on dengue viremia

	Placebo	Low-dose prednisolone	High-dose prednisolone	P value
AUC	20.96 (16.97 - 23.25)	21.29 (18.85 - 23.79)	21.56 (17.99 - 24.18)	0.8
Days from study enrolment to viremia clearance*	7 (5 - 8)	7 (5 - NE)	8 (5 - 8)	0.9

AUC: Area under curve of log viremia from day 3 to day 6 of illness.

** The values are expressed as median and inter quartile range. NE: not estimable*

5.3.3 NS1 kinetics

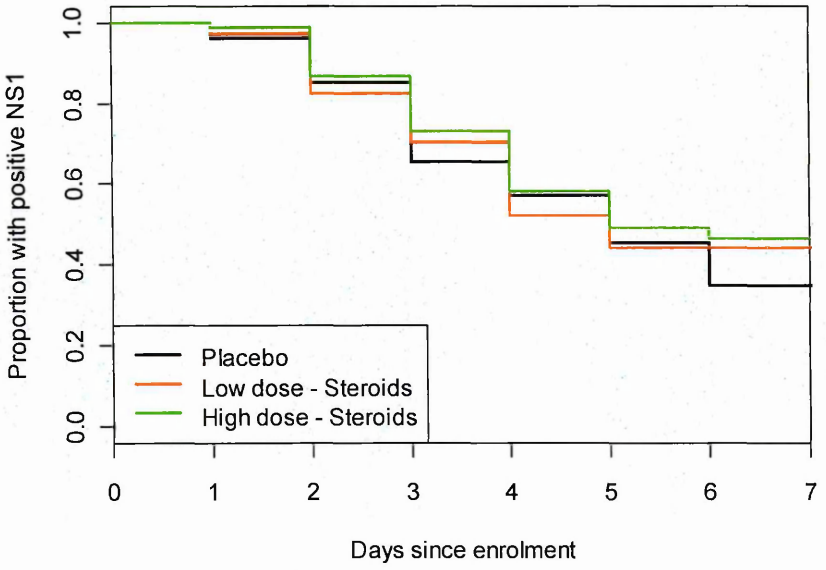


Figure 5-2: Kaplan-Meier plot of NS1. The figure shows a Kaplan-Meier plot indicating the proportion of patients remaining positive for NS1 in the three treatment groups on each day following study enrolment.

NS1 persisted for a long time in the dengue patients, and many patients in fact remained positive at discharge (Figure 5-2). The median (IQR) of days from enrolment to negative NS1 status was 5 (3 - 8) days for patients receiving placebo, 5 (3 - 11) days for those receiving high-dose prednisolone, and 5 (3 – not estimable) days for those on

low-dose prednisolone. There was a borderline trend between patients receiving prednisolone and prolongation of NS1 antigenaemia (p=0.08, multiple linear regression adjusted for day of illness at enrolment, immune status and serotype).

5.3.4 Differentially abundant gene expression linked to high-dose prednisolone therapy

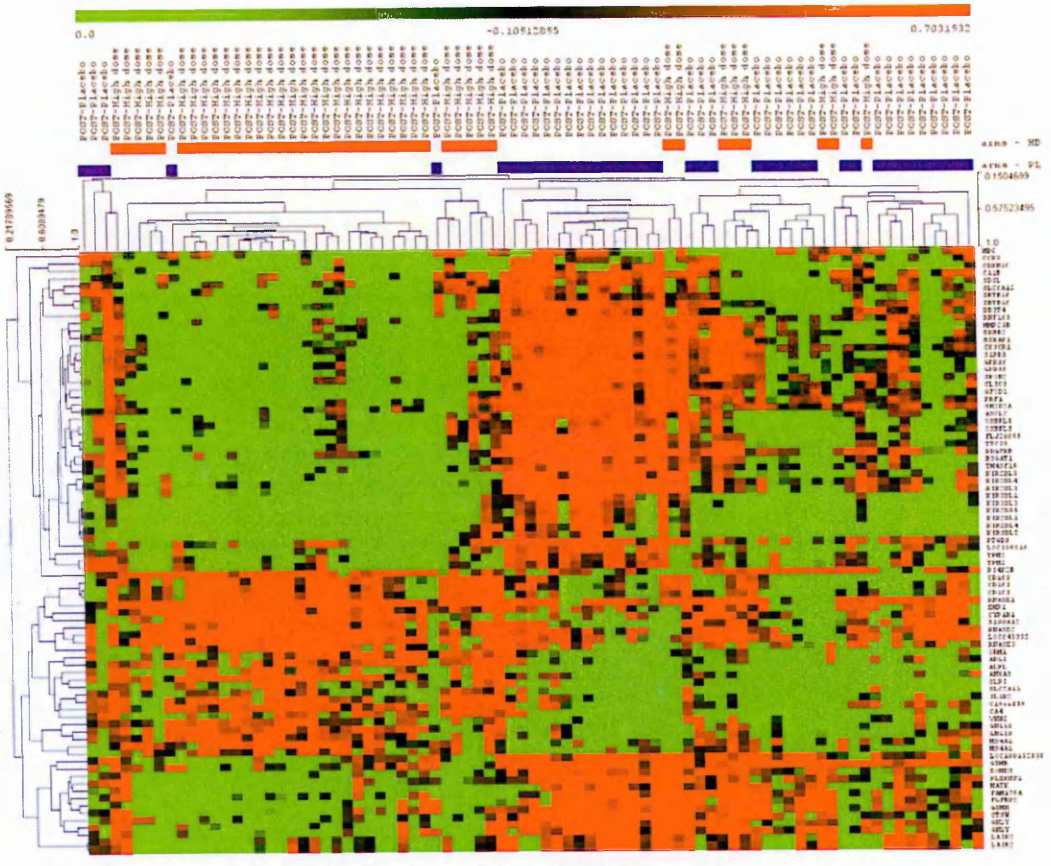


Figure 5-3: Unsupervised hierarchical clustering of the 81 differentially abundant elements distinguishing patients that received high-dose prednisolone from placebo treated patients 2 days after starting therapy. Individual patient samples (2 days after starting treatment) are shown in vertical columns while the 81 differentially abundant transcripts (from 67 genes) are shown in horizontal rows.

Microarray analysis was carried out in the first 123 consecutive patients enrolled in this study. At baseline there were no transcripts identified in the microarray that were

differentially abundant between the treatment arms. Similarly, there were no differentially abundant transcripts between treatment groups in late-convalescence (a median of 29 (IQR 27 - 30) days after enrolment) or between placebo and low-dose prednisolone patients 2 days after commencing treatment. In contrast, 81 differentially abundant transcripts (25 from 21 genes more abundant, and 56 from 46 genes less abundant) were detected when comparing whole-blood gene expression profiles between high-dose prednisolone and placebo-treated patients 2 days post-commencing treatment (median time 43hrs (IQR 42 - 43) for high-dose prednisolone and 43hrs (IQR 42 - 44) for placebo). In these samples the median time since the last dose of study medication was 23hrs (IQR 22 - 24) for high-dose prednisolone and 23hrs (IQR 22 - 23) for placebo. Unsupervised hierarchical clustering of the 81 elements that represent the high-dose prednisolone-associated signature is shown in Figure 5-3.

5.3.5 Validation by quantitative RT-PCR (qRT-PCR) of the high-dose prednisolone-associated gene signature

RT-PCR measurements were performed for 31 (46%) of the 67 genes (81 transcripts) on the basis of their having plausible roles in immune function. RT-PCR was performed on 600 whole blood RNA samples collected at baseline, 2 days after starting treatment, and at the follow-up visit from 223 (99%) of the 225 patients who were enrolled into the study.

RT-PCR measurements were highly concordant with the microarray findings in demonstrating differences in transcript abundance between high-dose prednisolone recipients and patients receiving placebo (Figure 5-4).

We extended these analyses by investigating a dose-response relationship in the prednisolone-associated gene expression signature. After adjustment for *a priori* defined baseline variables (day of illness at enrolment, absolute neutrophil and lymphocyte count, baseline transcript abundance) a highly significant dose-related affect on gene transcript abundance was observed for 31/31 transcripts using trend test (Table 5-3). These results collectively define a gene expression signature that is associated with high-dose prednisolone therapy in dengue patients.

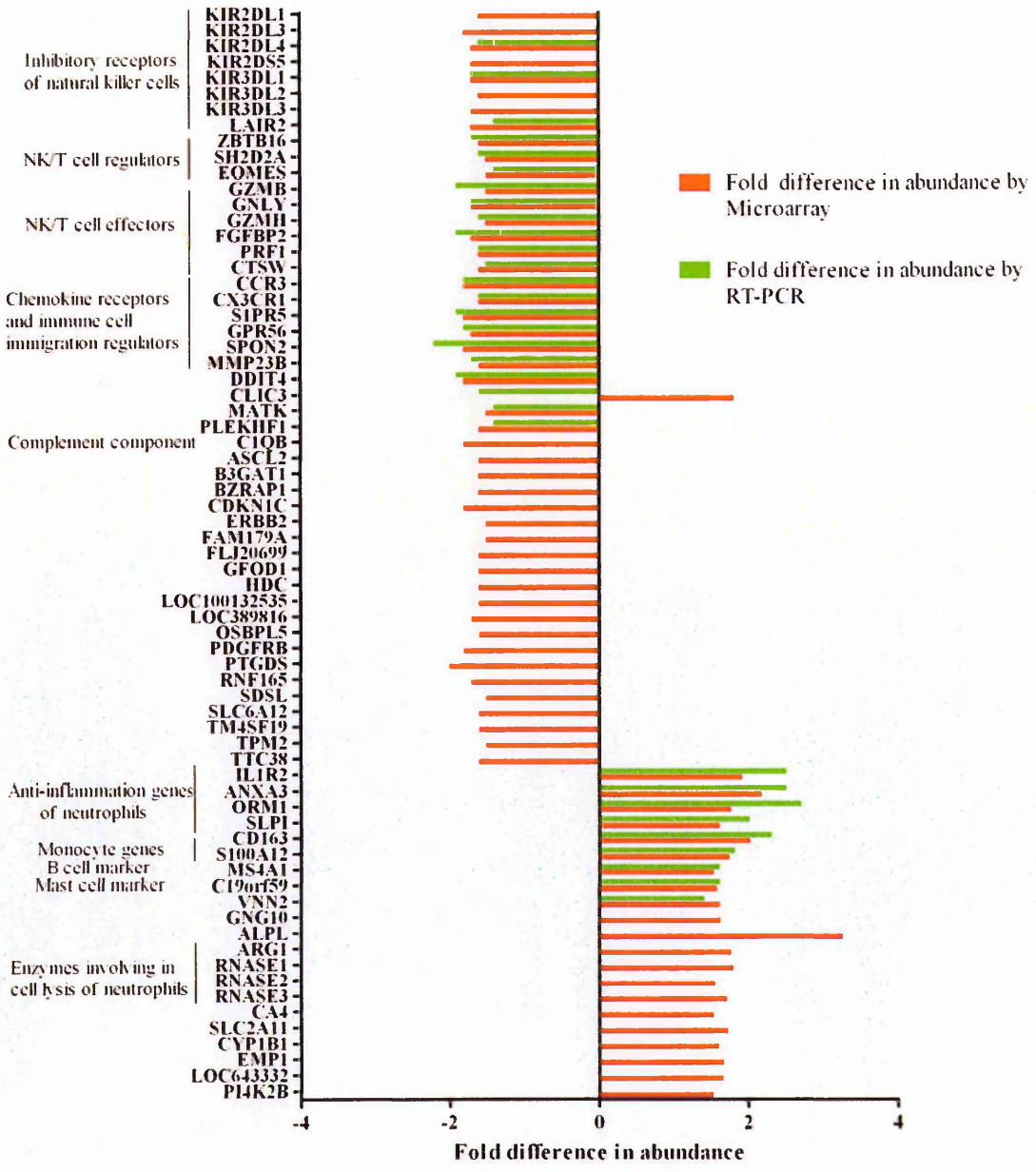


Figure 5-4: Fold difference in transcript abundance between high-dose prednisolone and placebo treated patients 2 days post-treatment. Shown is the ratio in abundance of transcripts measured by microarray and by RT-PCR from the 67 genes that distinguished high-dose prednisolone treated patients from placebo treated patients. Where results from multiple probes in the microarray were available for a single gene the mean result was used. Genes have been grouped and annotated according to their recognized biological functions.

Table 5-3: Genes with transcript abundance that were significantly different between patients treated with high-dose prednisolone and placebo

No	Gene Symbol	Microarray		RT-PCR		
		Abundance	Fold change	Relative expression ratio (95% CI) High-dose vs Placebo	Relative expression ratio (95% CI) Low-dose vs Placebo	Trend test - P value[a]
1	ORM1	Higher	1.8	2.7 (1.5 - 3.5)	NS	1.20E-06
2	ANXA3	Higher	2.2	2.5 (1.7 - 3.1)	1.4 (1.0 - 1.8)	5.01E-08
3	IL1R2	Higher	1.9	2.5 (1.5 - 3.1)	1.7 (1.0 - 2.1)	1.40E-07
4	CD163	Higher	2.0	2.3 (1.7 - 2.8)	1.5 (1.1 - 1.8)	2.77E-08
5	SLPI	Higher	1.6	2.0 (1.3 - 2.6)	NS	1.04E-04
6	S100A12	Higher	1.7	1.8 (1.1 - 2.2)	NS	6.54E-06
7	MS4A1	Higher	1.5	1.6 (1.2 - 1.8)	1.2 (1.0 - 1.4)	1.31E-05
8	C19orf59	Higher	1.6	1.6 (1.1 - 1.9)	NS	9.64E-04
9	VNN2	Higher	1.6	1.4 (1.1 - 1.7)	NS	2.78E-02
10	KIR3DL1	Lower	1.7	1.7 (1.4 - 2.6)	1.4 (1.1 - 2.1)	1.01E-03
11	KIR2DL4	Lower	1.7	1.6 (1.3 - 2.2)	1.3 (1.1 - 1.8)	3.88E-05
12	LAIR2	Lower	1.7	1.4 (1.2 - 1.8)	1.3 (1.1 - 1.6)	2.46E-03
13	ZBTB16	Lower	1.6	1.7 (1.4 - 2.2)	1.5 (1.3 - 1.9)	6.42E-07
14	SH2D2A	Lower	1.5	1.6 (1.3 - 2.0)	1.4 (1.2 - 1.8)	5.09E-04
15	EOMES	Lower	1.5	1.4 (1.2 - 1.9)	1.4 (1.2 - 1.7)	8.20E-03
16	GZMB	Lower	1.5	1.9 (1.5 - 2.6)	1.5 (1.2 - 2.0)	1.57E-04
17	GNLY	Lower	1.7	1.7 (1.4 - 2.2)	1.3 (1.1 - 1.7)	3.67E-04
18	GZMH	Lower	1.5	1.6 (1.3 - 2.2)	1.3 (1.1 - 1.8)	9.01E-04
19	PRF1	Lower	1.6	1.6 (1.3 - 2.2)	1.4 (1.2 - 1.9)	1.17E-03

20	CTSW	Lower	1.6	1.5 (1.2 - 1.9)	NS	1.26E-02
21	CCR3	Lower	1.8	1.8 (1.4 - 2.5)	1.4 (1.2 - 2.0)	5.63E-08
22	CX3CR1	Lower	1.6	1.6 (1.3 - 2.1)	1.3 (1.1 - 1.7)	1.16E-03
23	S1PR5	Lower	1.8	1.9 (1.6 - 2.5)	1.5 (1.2 - 1.9)	9.89E-06
24	GPR56	Lower	1.7	1.8 (1.5 - 2.6)	1.4 (1.1 - 2.0)	4.74E-04
25	SPON2	Lower	1.8	2.2 (1.9 - 3.0)	1.6 (1.3 - 2.2)	2.04E-07
26	DDIT4	Lower	1.8	1.9 (1.7 - 2.4)	1.5 (1.3 - 1.9)	5.67E-09
27	FGFBP2	Lower	1.7	1.9 (1.5 - 2.7)	1.6 (1.3 - 2.2)	2.06E-04
28	MMP23B	Lower	1.6	1.7 (1.4 - 2.4)	1.4 (1.2 - 1.9)	1.01E-03
29	CLIC3	Higher	1.8	1.6 (1.3 - 2.1)	1.3 (1.1 - 1.7)	3.55E-04
30	MATK	Lower	1.5	1.4 (1.2 - 1.8)	NS	7.13E-03
31	PLEKHF1	Lower	1.6	1.4 (1.2 - 1.8)	NS	6.41E-03

[a] *All p values were corrected with Benjamini-Hochberg method for multiple test correction.*
***NS** denotes genes that were not significantly different in gene expression between the patients receiving low-dose prednisolone and placebo and/or the confidence interval of the relative expression ratio (95% CI) contained 1.*

5.3.6 Phenotypic profile of T lymphocytes and response to prednisolone therapy

The phenotype (HLA-DR, CD38 and Ki-67) of T lymphocytes ($CD3^+4^+$ and $CD3^+8^+$) in fresh whole-blood samples collected 4-11 days after commencing treatment was determined in 100 consecutive patients. The baseline characteristics of these patients are described in Table 5-4 The percentage of $CD8^+Ki67^+$ T cells was increased in patients treated with prednisolone (p=0.02 for low-dose prednisolone and 0.03 for high-dose prednisolone versus placebo, Mann-Whitney test, Figure 5-5). The percentage of $CD8^+CD38^+$ T cells was also significantly elevated in the low-dose prednisolone group but not in the high-dose prednisolone group compared to the placebo patients (p=0.01, Mann-Whitney test, Figure 5-5). However, these associations were not significant after adjustment for multiple testing using the Benjamini-Hochberg correction.

Table 5-4: Baseline characteristics of patients included in T cell phenotypic analysis

	Placebo (N=38)	**Low-dose (N=31)**	**High-dose (N=31)**
Sex [Male]	29 (76)	21 (68)	24 (77)
Age [years]	15 (13 - 17)	14 (12 - 17)	14 (12 - 16)
WBC [10^6 dL]	5.4 (4.2 - 6.9)	5.2 (4.5 - 6.1)	5 (4.1 - 5.7)
Lymphocyte [10^6 dL]*	42.9 (37 - 48.3)	46.5 (41.4 - 53.4)	43.1 (38.4 - 49.7)
Day of illness of sample (days)	9 (8 - 10)	8 (8 - 9)	8 (8 - 9)
Serotype			
DENV1	13 (34)	18 (58)	18 (58)
DENV2	23 (61)	8 (26)	7 (23)
DENV3	2 (5)	4 (13)	5 (16)
DENV4	0	1 (3)	1 (3)
Immune status**			
Primary	6 (16)	7 (23)	9 (29)
Secondary	25 (66)	13 (42)	12 (39)

*Numbers are median (IQR) or N (%). No significant difference was found between the groups. *5 missing data. ** 28 missing (7, 11 and 10 patients respectively in the three treatment arms).*

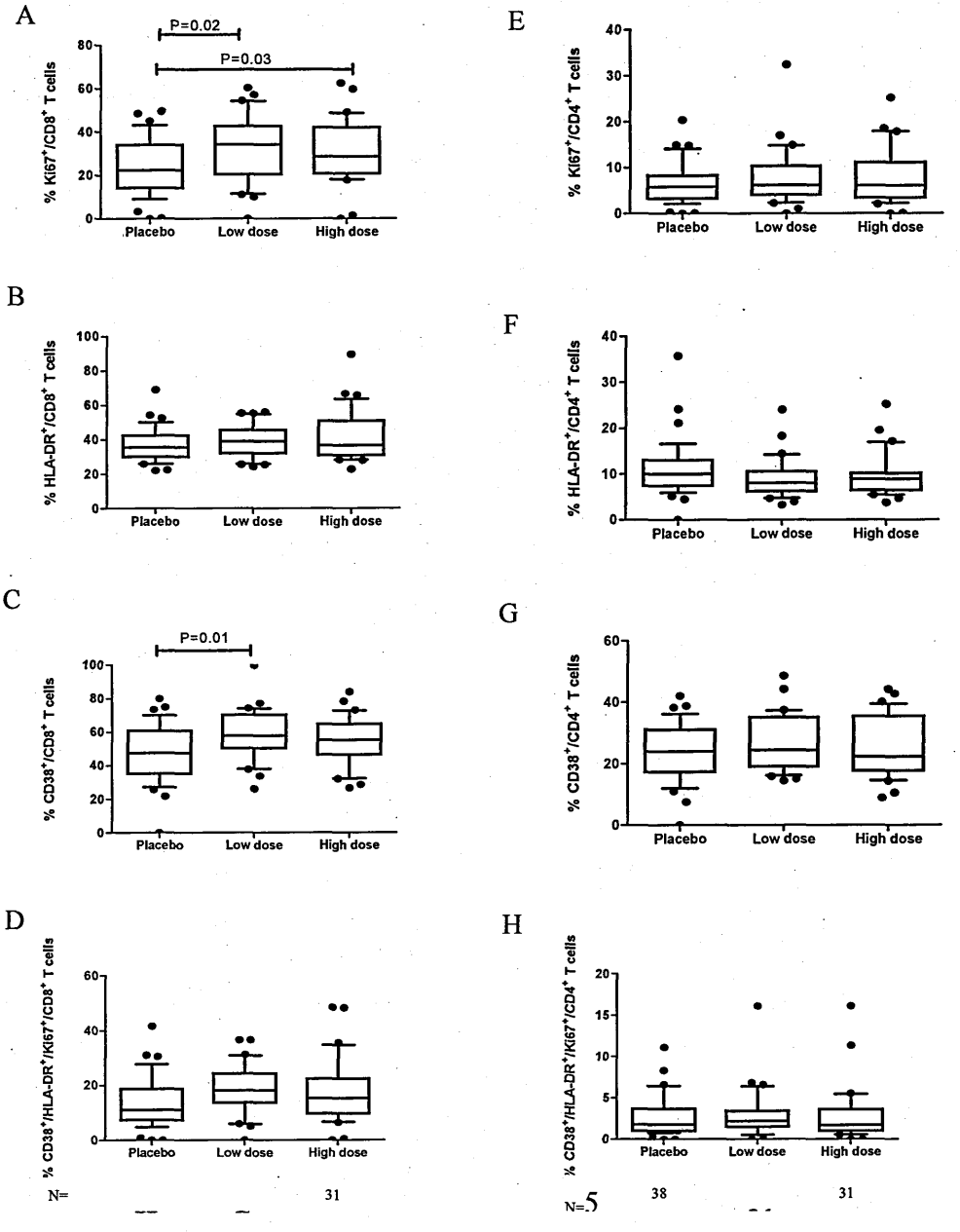


Figure 5-5: Frequency of activated CD8+ (A - B - C - D) and CD4+ (E - F - G - H) T cells in peripheral blood at discharge. Shown in each panel is the median ± IQR that represents the percentage of T cells positive for Ki67 (A, E), HLA-DR (B, F), CD38 (C, G) and triple positive for CD38/HLA-DR/Ki67 (D, H). The number of patients evaluated is indicated below D and H.

5.3.7 Plasma cytokines and prednisolone therapy

Concentrations of 11 cytokines and chemokines were measured in 636 serial plasma samples from 223 patients at three time-points: at baseline, 2 days after commencing treatment, and at follow-up in late convalescence. Follow-up samples were missing from 30 patients (placebo - 10; low-dose prednisolone - 8; and high-dose prednisolone - 12). Although within the detectable range in 98% of samples tested, the cytokine/chemokine concentrations (adjusted for pre-treatment values and day of illness at enrolment) were neither significantly elevated nor significantly different between the treatment groups 2 days after starting therapy (Table 5-5). An unadjusted comparison of values gave similar profiles. Since these findings were unexpected, the accuracy of the Bio-plex immunoassay was confirmed by repeating the IFN-γ measurement on 72 patients randomly chosen from 223 patients with serial samples, using a conventional sandwich ELISA technique (R&D Systems - USA). Near identical results were found (See Figure 5-6).

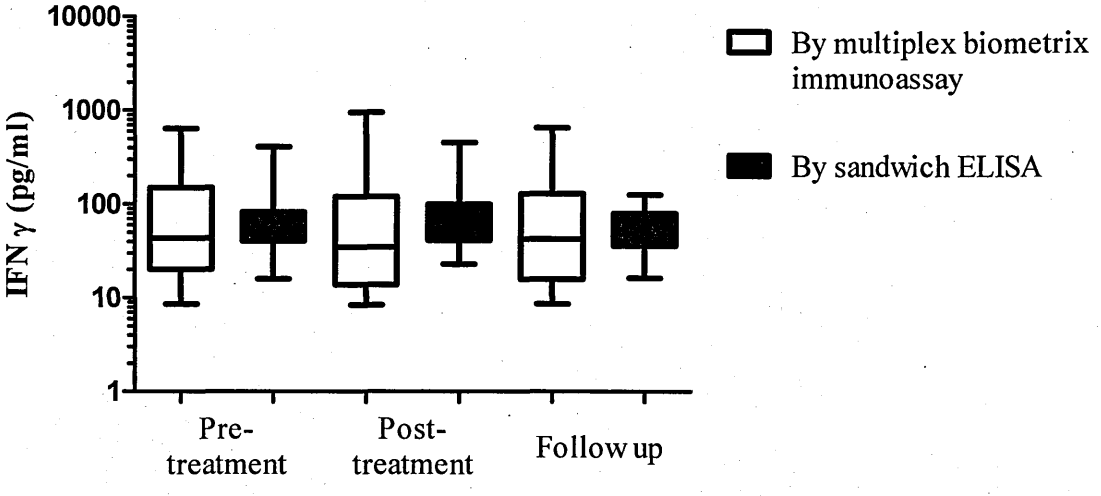


Figure 5-6: Comparisons of plasma IFN-γ levels measured by two methods: multiplex biometrix immunoassay and sandwich ELISA. 72 patients with serial samples were randomly chosen from 223 patients for IFN-γ sandwich ELISA (R&D Systems, USA) and compared with multiplex biometrix immunoassay. No significant differences were found between the two methods or between the sample time-points.

Table 5-5: Plasma cytokine concentrations 2 days post initiation of treatment

Cytokine	Plasma concentration 2 days after starting therapy			Trend test	
	High-dose prednisolone	Low-dose prednisolone	Placebo	Multiplicative effect (95%CI)	Adjusted P
IL-1β	11.3 (8.5 - 15.1)	12.5 (10.7 - 16.3)	13.1 (10.2 - 18.3)	0.90 (0.75 - 1.07)	0.24
IL-2	50.6 (37.6 - 66.6)	54.6 (40.2 - 69.5)	50.9 (38.4 - 74.6)	0.89 (0.72 - 1.11)	0.31
IL-4	13.4 (10.2 - 17.2)	14.1 (10.2 - 16.9)	13.3 (10.0 - 19.0)	0.91 (0.77 - 1.08)	0.29
IL-5	79 (62.6 - 101.1)	79 (63.3 - 92.0)	79.3 (64.6 - 96.4)	0.91 (0.73 - 1.14)	0.42
IL-6	132.9 (93.3 - 183)	125.7 (106.4 - 162.4)	140.1 (102.3 - 199.6)	0.98 (0.80 - 1.20)	0.83
IL-10	119.4 (80.8 - 166)	118.2 (96.7 - 152.6)	126.7 (98.4 - 167.4)	0.98 (0.92 - 1.05)	0.65
IL-12p70	24 (16.0 - 32.4)	23.1 (16.0 - 30.1)	23.2 (15.8 - 30.2)	0.97 (0.82 - 1.14)	0.70
IL-13	26.4 (17.3 - 40.1)	23.6 (17.1 - 40.1)	25.6 (18.5 - 36.1)	0.94 (0.80 - 1.10)	0.42
IFN-γ	56.5 (41.0 - 96.0)	58.1 (42.4 - 88.5)	62.2 (41.8 - 85.3)	0.97 (0.87 - 1.06)	0.48
TNFα	13.2 (10.6 - 17.6)	12.7 (10.2 - 16.2)	14.5 (10.0 - 18.1)	0.95 (0.780 - 1.13)	0.56
IP-10	8398 (5099.2 - 11506.5)	8097.5 (4998.9 - 11424.4)	8099 (4701.8 - 10630.3)	1.06 (0.98 - 1.14)	0.14

All data are presented as median (IQR) values.

5.3.8 Serological responses and prednisolone therapy

DENV-reactive IgM and IgG levels in early convalescence (days 4-11 post-treatment) were not significantly affected by prednisolone therapy (Figure 5-7). In a subset of patients (n=75) who returned for a 6-month follow-up visit, $PRNT_{60}$ titers to DENV1-4 were determined with plasma collected at that visit. No difference was observed between treatment groups with respect to the $PRNT_{60}$ titers to each patient's autologous serotype. As expected, in many cases the highest plasma titer in each patient was not to the serotype responsible for their recent infection. Collectively, these results suggest the antibody response to DENV infection is not impaired by prednisolone therapy.

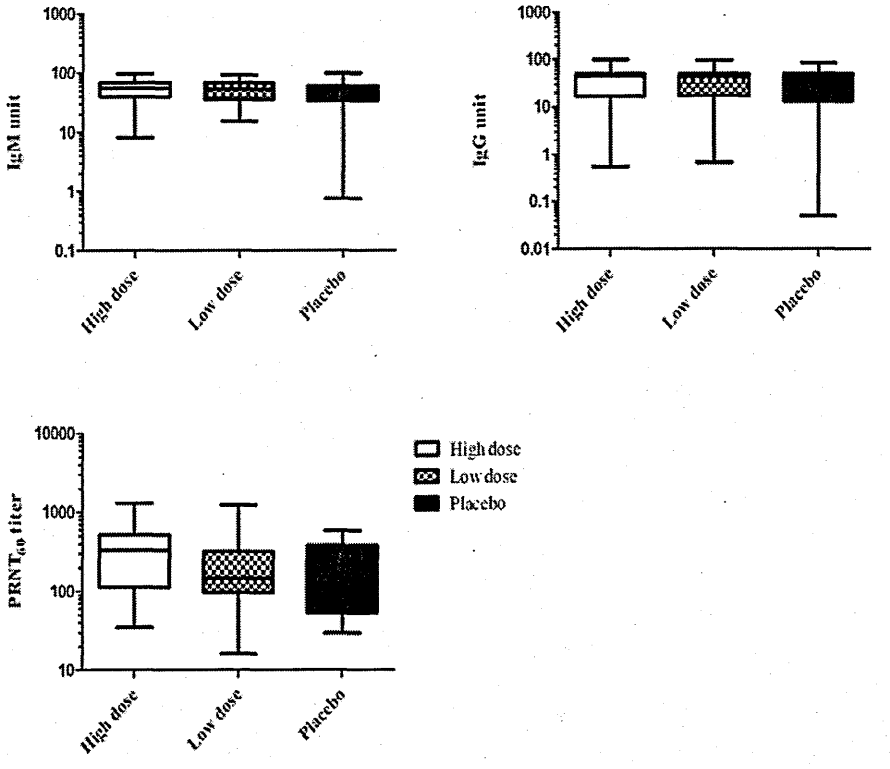


Figure 5-7: Serological responses and prednisolone therapy. Comparisons of A. IgM at discharge; B. IgG at discharge (4-11days post-treatment); and C. $PRNT_{60}$ values 6 months after illness. No differences were found between the treatment groups

5.4 Discussion

A host pro-inflammatory immune response is widely believed to contribute to the increased capillary permeability associated with dengue infections. Therapeutic agents such as corticosteroids might attenuate this host response and thus provide clinical benefits. The current study was linked to a randomized controlled trial of early prednisolone therapy for dengue and focused on the effect of prednisolone on virological factors. The results emphasize the prednisolone safety profile by showing that prednisolone did not increase dengue viremia or prolong NS1 antigenemia in patient plasma. In addition, the work also provides insight into the poor therapeutic efficacy of prednisolone by identifying only a small prednisolone-associated footprint (just 81 transcripts differentially abundant from 47,231 evaluated) on the whole-blood gene expression profile. Moreover, neither acute-phase plasma cytokine concentrations nor the percentage of activated T cells in early convalescence were measurably attenuated by prednisolone treatment. The limited immunomodulation achieved by prednisolone is consistent with it having negligible measurable benefits in the clinical trial on which this work is based.

There is extensive clinical experience of using corticosteroids in the management of non-acute, non-infectious inflammatory diseases. Corticosteroids are believed to deliver clinical benefits in these inflammatory conditions via genomic and non-genomic pharmacological actions that result in inhibition of transcription of pro-inflammatory genes and an increase in the transcription of anti-inflammatory genes [187]. Corticosteroids also elicit apoptosis in activated human T cells and can suppress the virus-induced production of type I IFNs and modulate their downstream signaling [188, 189]. Against this backdrop, where the immunomodulatory actions of corticosteroids are well established, it was surprising that stronger signals of immune-modulation were not observed in this current study. The absence of a measurable impact of prednisolone on plasma cytokine concentrations two days after enrolment reflects the absence of any significant elevation of the 11 investigated cytokines in the acute phase compared to convalescence. This is at odds with a body of literature indicating elevated plasma/serum cytokine concentrations are a prominent feature in

the host response [88]. However one limitation of this work, in comparison to other studies is that cytokine/chemokine concentrations were not measured in serial daily plasma specimens and therefore may have missed transient changes in particular markers [38, 86].

Although corticosteroids are recognized for their pro-apoptotic effect in leukocytes [188, 190], there was no evidence for a prednisolone-associated reduction in $CD4^+$ or $CD8^+$ T cells bearing surface activation markers during early convalescence. Since the study involved only 100 consecutive patients, it is plausible it was underpowered to detect subtle differences in T cell responses between treatment groups. However, the absence of any measurable effect in this sample size suggests the impact of prednisolone on the characteristics of the blood T cell population that was investigated is not large. In the serology assays the total anti-dengue IgM and IgG levels do not differentiate between serotype specific and serotype cross-reactive antibodies, which have been reported to be different in their neutralizing abilities [191]. However, they still reflect the overall antibody response in dengue, and yet there was no apparent effect of prednisolone in this study, with similar levels of capture IgG and IgM on the discharge day as well as similar PRNT titers 6 months after the onset of the acute illness.

At the transcriptional level, there were changes in the whole-blood host gene expression profile in patients that had received 2mg/kg prednisolone (but not the lower dose, 0.5 mg/kg) compared to placebo-treated patients. A striking finding was the under-abundance of transcripts representing granzyme B (GZMB), granzyme H (GZMH), granulysin (GNLY), perforin (PRF1), Ksp37 (FGFBP2) and cathepsin W (CTSW), each of which is associated with the secretory and cytolytic activities of T and NK cells. This was independent of the blood lymphocyte count. Furthermore, transcripts from eomesodermin (EOMES: a transcriptional regulator required for full effector differentiation of $CD8^+$ T cells and NK cells) and SH2D2A which encodes TSAd (a positive regulator of proximal T cell receptor signal transduction) were also under abundant in prednisolone treated patients [192, 193]. High-dose prednisolone treatment was also associated with an under-abundance of transcripts from genes

belonging to the leukocyte associated Ig like receptor 1 (LAIR1) and the killer-cell immunoglobulin-like receptor family (KIR3DL1, KIR2DL4). These genes are involved in the regulation of NK cell activity to prevent recognition of self, and their under-abundance might suggest dysfunction of normal NK cell responses during prednisolone therapy. Prednisolone treatment was associated with elevated transcripts encoding decoy receptor of interleukin 1 (IL1R2), the endocytic receptor for hemoglobin–haptoglobin (CD163) and orosomucoid 1 (ORM1), all of which are known to be corticosteroid induced [194-197]. These proteins are produced by neutrophils or monocytes/macrophages and take part in anti-inflammatory reactions. Moreover, each has been reported to interfere directly or indirectly with the proliferation and cytolytic response of T cells [198-201]. Taken together, the diminished abundance of transcripts encoding T and NK effector proteins might suggest impaired anti-viral cytolytic responses during high-dose prednisolone therapy. However viremia levels were not significantly higher or more prolonged in prednisolone-treated patients, nor did these patients have other hematological or biochemical laboratory abnormalities that would suggest impaired viral clearance. It is plausible that the lower transcript abundance of these elements in whole blood is not biologically significant in terms of resolving infection and that other components of the immune response, such as antibodies or complement, are more important and/or compensatory.

Although quite comprehensive in approach, there are some limitations to the work presented here. Previously, *in vitro* studies have shown prednisolone to have a significant effect on complement activation [202, 203], and in other clinical studies, prednisolone has been shown to provide benefit in rheumatoid arthritis and lung or cardiac surgery by inhibiting complement activation [204-207]. In this study, although I found down regulation of the gene encoding for C1QB in high-dose patients compared with placebo recipients, I did not find alterations in other components of the complement system. Unfortunately I was unable to confirm this result by qRT-PCR due to limited remaining samples. Neither was I able to assess complement activation although there is good evidence that complement is consumed and split products generated in the course of dengue, and that this may be important to pathogenesis

[68]. Secondly the sampling schedule may have missed transient but important differences in cellular gene expression signals between patients in different treatment arms. And finally prednisolone may have important actions in tissues that are not revealed in whole blood.

Nevertheless these results, coupled with the findings from the clinical trial, suggest that early prednisolone therapy has little impact on the host immune response or the clinical evolution of dengue. One possible explanation is that early prednisolone therapy is "too little, too late" to attenuate the infection-driven processes that lead to the altered capillary permeability, thrombocytopenia, and haemostatic derangements. Even earlier treatment, or possibly higher dose therapy, might have led to a greater prednisolone impact on the immune response and the clinical/laboratory phenotype. However, enrolling into this trial was quite challenging (with around one third of patients enrolled on day 2, and two thirds enrolled on day 3 of illness), and attempting to enroll patients prior to day 2 of illness would be even more difficult. Use of higher dose therapy would be problematic since a small number of subjects on the 2mg/kg dose developed hyperglycemia, a well recognized complication of steroid therapy, and it is unlikely that clinicians would consider this an acceptable risk to take with a treatment eventually intended to be administered to large numbers of patients at the community level.

Notwithstanding these limitations, the results described here underscore the challenge of modulating an immune response that has been driven by days of DENV replication in host tissues. More fundamentally, these results are a reminder that although immune-driven pathophysiological changes are good candidates to explain capillary permeability, the precise causal mechanisms remain poorly understood. This represents a major knowledge gap in our understanding of disease pathogenesis that also undermines development of specific therapeutic interventions in the future.

Chapter 6

GENERAL DISCUSSION AND FUTURE POSSIBILITY

This thesis makes several significant steps towards developing a better understanding of dengue pathogenesis. First, I established all-inclusive models based on both in-house and commercial serological tests to define immune status, a well established factor known to influence pathogenesis. Advantages of these models compared to the current systems include convenience of sampling (single specimens taken during the acute illness) and high accuracy (80 – 85%), as well as the use of relatively simple serological techniques. In particular the model based on the Panbio Indirect IgG ELISA gave the best performance (accuracy of 85%), and potentially could be widely applied in dengue pathogenesis studies, capitalizing on the high reproducibility of this commercial kit. To date there has been considerable variability in how immune status is defined in pathogenesis studies. Both HI and PRNT, currently regarded as the gold standard for defining immune status, are complicated and/or time consuming to perform and are not standardized between different laboratories around the world. Similarly algorithms based on in-house serological assays vary considerably between laboratories. Although use of commercial kits is constrained by their high cost, consistency is generally good both between centers and over time.

The all-inclusive model based on the Panbio Indirect IgG performed better in the early phase, before Day 5 of illness. For assessing dengue patients during the late acute phase, the good performance characteristics of our in-house capture IgG and the in-house capture IgM/IgG ratio are encouraging. For more widespread use similar algorithms based on commercial capture IgM and IgG kits are likely to perform well and should be evaluated formally in a similar way. It would also be valuable to assess the generalizability of the models I have developed in other epidemiological settings with different endemicity and to include evaluation of the possible effects of other flaviviruses.

From a clinical perspective since secondary dengue is a risk factor for severe disease and the all-inclusive model based on Panbio Indirect IgG was able to define immune status with high accuracy very early in the course of illness (Day 2) based on a single specimen, it is possible the algorithm might also be useful in clinical practice. The test would need to be combined with an effective test to also make a definitive diagnosis of dengue at this time. Modern PCR techniques are now very sensitive, and both formal NS1 ELISAs and NS1 rapid tests can be used for this purpose, albeit with certain limitations on sensitivity. Using this kind of combined approach it might be possible to decrease the number of patients currently hospitalized for close monitoring, thus decreasing the overall burden on healthcare systems where dengue is endemic. Similarly it might be possible to use this strategy to target enrolment to intervention trials of potential dengue therapeutics to the patient population most at risk of developing severe disease.

Second, I confirmed and expanded the current knowledge base regarding the influence of immune status and serotype on the magnitude and kinetics of dengue viremia, by carrying out detailed assessments on almost 900 patients infected with DENV1, DENV2, and DENV3. In our laboratory in-house IgG and IgM capture ELISAs are used routinely to process very large numbers of specimens each year; I applied the model based on the in-house capture IgM/IgG ratio to define the immune status of these patients and measured plasma viremia daily throughout the acute illness. I found clear differences in viremia kinetics between the 3 serotypes assessed, with viremia typically being highest in DENV1 and lowest in DENV3 infections. I confirmed the higher viremia associated with primary DENV1 infections compared to secondary DENV1 infections that was reported previously by our group, and was also able to examine the effect of immune status on viremia in DENV2 and DENV3 infections. Similar to DENV1 I found higher viremia in primary compared to secondary DENV3 infections but I found no difference in viremia between primary and secondary DENV2 infections.

The finding of generally higher viremia in DENV1 infections is interesting, particularly in view of the fact that DENV1 has been the predominant serotype

circulating in southern Vietnam for almost a decade from 2006 until now. If DENV1 has better fitness than other serotypes, resulting in higher viremia, this may offer a competitive advantage and potentially alter transmission dynamics since plasma viremia is known to influence the likelihood of developing a viable infection in the mosquito vectors. The clear differences in viremia patterns between the three serotypes are also intriguing. One limitation is that we do not know anything about viremia kinetics before the onset of symptoms – although we enroled the study participants early in the illness course viremia was almost always already declining by this time. Further work to try to understand the contribution of viremia kinetics and immune status in the presymptomatic phase using mathematical modeling may prove informative, although many assumptions must be made and there is always uncertainty about whether modeling findings really reflect the biological facts. Another potential source of information would be from challenge studies using non-human primates. However relationships between findings in animal models and what happens in human disease are also not known, and results from such studies must also be interpreted with caution. Human challenge studies using wild-type viruses were performed in endemic populations many decades ago, and may be a useful methodology to revisit to try to understand dengue pathogenesis, particularly since sophisticated modern techniques to assess virological and immunological responses could be applied to samples obtained in the presymptomatic phase. However, the ethics and safety of such studies needs to be carefully considered, as well as the practicalities of performing challenge studies in endemic settings where participants may be bitten by infected mosquitoes anyway.

This is the first time an association between higher viremia in the early acute phase (Day 3) and the risk for subsequent development of DSS has been identified. Although some previous studies have found associations between viremia and dengue severity assessed in terms of DF versus DHF, the findings have not been consistent across studies, probably because sample sizes have generally been small and the natural evolution of viremia during the illness course has not always been accounted for. Clear differentiation between DF and DHF is also difficult without very stringent clinical data collection, while DSS is much more straightforward to identify. The

findings from this work strongly support viremia as an important factor in determining dengue pathogenesis, and reinforce the view that viremia is an important endpoint for use in clinical trials of dengue therapeutics. Similarly, as higher viremia on Day 3 was associated with progression to dengue shock, this could be considered for incorporation into clinically relevant algorithms designed to identify high risk patients and influence decision-making about whether to triage a particular individual for admission or observation as an outpatient.

In addition to trying to understand dengue pathogenesis, considerable efforts are being made by the dengue research community to advance strategies to control dengue, ideally by developing a safe and effective vaccine or by eliminating dengue using novel vector control methods such as the mosquito parasite, *Wolbachia.* However even though these programs may be successful in the long term, effective treatment for dengue is still required now. A number of anti-viral agents have been trialed and shown to be safe, but unfortunately to date none of these agents were successful in reducing plasma viremia or in preventing the development of clinical complications. Our group recently assessed the safety of an alternative approach, using immune modulation with oral prednisolone early in the disease course, but also failed to demonstrate clinical efficacy. The third main area of research presented in this thesis relates to the work I did for this clinical trial to demonstrate prednisolone's safety profile by showing that it did not augment or lengthen the existing plasma viremia or NS1 antigenaemia. I also investigated the effects of early prednisolone therapy on the transcriptional profile of a range of immunological correlates, but could only identify a small prednisolone-associated footprint – only 81 transcripts were differentially abundant between the prednisolone and placebo recipients among the 47,231 that I evaluated. Also there was no significant effect of prednisolone on cytokine levels, T cell phenotypes or antibody responses. It is possible that these findings reflect the timing of the prednisolone therapy; even though treatment was commenced within 72 hours of fever onset this may still be too late to alter the trajectory of a disease course established even before the onset of symptoms. Alternatively the dose of prednisolone may have been too low to influence the immune response, but since some patients receiving the higher prednisolone dose in the trial already experienced adverse effects

it would not be feasible to increase it. Another explanation of the poor effectiveness of prednisolone is that the treatment was not directed at the appropriate population. Usually, dengue is a relatively mild disease, with most patients recovering spontaneously after several days of fever and only a small proportion of cases developing severe disease. Prednisolone may have little or no effect on these mild patients, and this may have diluted any real prednisolone effect in our trial. If the trial could be conducted on patients who were more likely to progress to severe dengue, it is possible prednisolone might have shown better effect. However, in order to identify such patients a good prognostic algorithm is required, and at present no suitable algorithm has been identified. Work is ongoing in a very large multicentre observational study to try to develop an effective prognostic algorithm, and it is possible that a) inclusion of a method to identify secondary dengue cases in the early acute phase, and b) measurement of plasma viremia within 72 hours, may both contribute to such an algorithm.

Although early prednisolone therapy failed to show efficacy in this trial, the results confirm that our understanding of dengue pathogenesis, especially how immune responses alter the pathophysiology of disease, in particular the severity of capillary permeability, is poor. At present our understanding about dengue immuno-pathogenesis is mainly derived from in-vitro studies or studies using animal-models, and interpretation of these findings needs careful consideration. Pathogenesis studies on humans will become increasingly important if we are to improve our understanding of this complex disease.

In summary, in this thesis I have presented the following results from my work focused on assessing plasma viremia and host immune responses in dengue, aiming to better understand disease pathogenesis:

- I developed a number of models based on both in-house (capture IgG, capture IgM/IgG ratio) and commercial (Panbio Indirect IgG) assays that could differentiate primary from secondary dengue, using single specimens obtained on any day of illness during the acute phase with high

accuracy. The model based on the Panbio Indirect IgG could be applied in future studies of pathogenesis across a variety of settings, allowing comparisons between different laboratories and research groups across the world, while the models based on the in-house capture IgG assay and in-house capture IgM/IgG ratio are very useful for studies performed locally by our group in Ho Chi Minh City.

- I expanded the current knowledge base regarding the influence of immune status and serotype on the magnitude and kinetics of plasma viremia in almost 900 patients covering the spectrum of clinical dengue cases seen in our setting. Viremia was lower in secondary than primary DENV1 and DENV3 infections, but it was not significantly affected by immune status in DENV2 infections. Viremia was also generally higher in DENV1 infections compared to the other serotypes, potentially reflecting better fitness of this serotype. Higher viremia on Day 3 of illness was more likely to be associated with worse clinical outcomes, including a lower platelet nadir, greater haemoconcentration, and development of shock.
- Although early therapy with prednisolone did not have a demonstrable clinical effect on patient outcomes in our recent clinical trial, its use during the phase of active viral replication was shown to be safe by demonstrating that the drug did not lengthen or increase plasma viremia or NS1 antigenaemia. In addition, there was a rather limited effect of prednisolone on the whole blood transcriptional profile and on immune responses (cytokine levels, T cell phenotypes and antibody responses), in agreement with the lack of a measurable clinical benefit in the trial.

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